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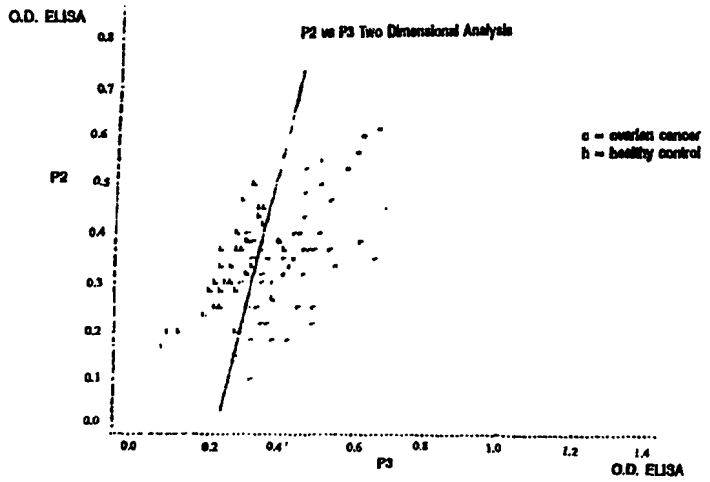
(54) Title: A WHOLE BLOOD/MITOGEN ASSAY FOR THE EARLY DETECTION OF A SUBJECT WITH CANCER AND KIT



(57) Abstract

This invention provides a method of detecting a subject having ovarian or breast cancer, comprising the following steps: a) obtaining a whole blood sample from the subject; b) incubating the whole blood sample in a culture in the presence of a media containing a mitogen with or without antigen, so as to induce polyclonal and specific activation of lymphocytic cells leading to antibody production; c) exposing the resultant culture of step b) to a specific tumor antigen, thereby allowing an antigen–antibody immune complex to form; d) detecting the antigen–antibody immune complex of step c); wherein the presence of tumor antigen associated antibody is indicative of the subject having the cancer.

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A WHOLE BLOOD/MITOGEN ASSAY FOR THE EARLY DETECTION OF A SUBJECT WITH CANCER AND KIT

BACKGROUND OF THE INVENTION

Currently available diagnostics for subjects having cancer includes assays based on recombinant protein and/or synthetic peptide-based antibody assays, and gene amplification techniques. The present invention provides a simple and early initial screening or detection of a tumor in a subject via the immune system.

Cancer or tumor cells emerge from the "normal" flora of cells in the body (both human and any other animals known to have tumors). Becoming cancerous/tumorigenic involves cellular changes. While these changes start off as mutations in the genetic code of the cells, the changes in the behavior of the cell stem from changes in protein expression levels. As the tumor grows, its suppresive effect on the immune system becomes stronger, thus active secretion of antibodies might be very low or even barely detectable.

Structures that are unique to the tumor cell are "tumor antigens". Structures that are not unique to the tumor cell yet might be expressed differently or in access amounts on it are considered "tumor associated antigens". Sometimes it is the "coating" of the protein with carbohydrates or lipids that determines its antigenicity. If we take for example the MUCIN from the mammary glands, the protein does not change in the tumor cell, yet antibodies are found against it in patients with breast cancer (and sometimes ovarian cancer too). The reason for it, most probably, is that the protein in the normal cell is not exposed at all due to a dense and thick coat of carbohydrate chains (ie. the protein is very heavily glycosylated). In the tumor cell glycosylation is incomplete thus leading to newly exposed protein sequences that serve (or can serve) as new antigens to the immune system. The present invention uncovers the potential of the humoral arm

of the immune system which enables detection of the tumor specific antibodies even if production of antibodies are suppressed *in vivo*.

SUMMARY OF THE INVENTION

The present invention relates to an improved assay for detecting a subject with cancer or infected with carcinogenic virus. This invention is contemplated for use as: 1) a diagnostic to determine if an individual has cancer; 2) a prognostic indicator, 3) a method to monitor the effectiveness of anti-cancer treatment (or the effectiveness of a new potential anti-cancer drugs or agents); 4) a method to determine re-occurrence after removal or therapy of a tumor in a subject; and 5) a method to detect and quantitate cancer in assays in clinical and research laboratories.

The present invention is useful as a screening assay for the detection of a tumor and a subject having a tumor or cancer in a subject. Antibodies derived are used for enhanced imaging and therapy.

This invention provides an *in vitro* method for the detection of tumor antigen associated antibodies in a sample obtained from a subject, comprising the following steps: a) obtaining a whole blood sample from the subject; b) incubating the whole blood sample in a culture in the presence of a mitogen with or without antigen containing media,so as to induce polyclonal and specific activation of lymphocytic cells leading to antibody production; c) exposing the resultant culture of step b) to a tumor antigen, thereby allowing an antigen-antibody immune complex to form; d) detecting the antigen-antibody immune complex of step c); wherein the presence of tumor associated antibodies is indicative of the subject having cancer.

BRIEF DESCRIPTION OF DRAWINGS

FIGURE 1: P3 - One dimensional analysis.

FIGURE 2: P3 and P2 - Two dimensional analysis

FIGURES 3A-3B: P1 and P3, P2 and P3 - Two dimensional analysis

FIGURES 4A-4F: Detection of SIV specific antibodies in seronegative mangabeys. Figure 4A shows that the level of O.D. reading on ELISA (post culture) can differentiate between true negative, seropositive, and "silent infection/exposure". Figures 4B-4F are a ratio of O.D. sample/O.D. negative control.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to an improved assay for detecting a subject with cancer or infected with carcinogenic virus. This invention is contemplated for use as: 1) a diagnostic to determine if an individual has cancer; 2) a prognostic indicator, 3) a method to monitor the effectiveness of anti-cancer treatment (or the effectiveness of a new potential anti-cancer drugs or agents); 4) a method to determine re-occurrence after removal or therapy of a tumor in a subject; and 5) a method to detect and quantitate cancer in assays in clinical and research laboratories.

This invention provides an *in vitro* method for the detection of tumor antigen associated antibodies in a sample obtained from a subject, comprising the following steps: a) obtaining a whole blood sample from the subject; b) incubating the whole blood sample in a culture in the presence of a mitogen with or without antigen containing media,so as to induce polyclonal and specific activation of lymphocytic cells leading to antibody production; c) exposing the resultant culture of step (b) to a tumor antigen, thereby allowing an antigen-antibody immune complex to form; d) detecting the antigen-antibody immune complex of step (c) wherein the presence of tumor associated antibodies is indicative of the subject having cancer.

In one embodiment, a sample of leucocytic cells is obtained from the subject and incubated in a culture in the presence of a media containing a mitogen with

or without antigen, so as to induce polyclonal and specific activation of lymphocytic cells. In another embodiment a sample of lymphocytic cells is obtained from the subject and incubated in a culture in the presence of a media containing a mitogen with or without antigen, so as to induce polyclonal and specific activation of B lymphocytic cells. In another embodiment a sample of PBMC is obtained from the subject and incubated in a culture in the presence of a media containing a mitogen with or without antigen, so as to induce polyclonal and specific activation of B lymphocytic cells.

Types of cancer/neoplasms include but are not limited to: myeloid leukemia such as chronic myelogenous leukemia, acute myelogenous leukemia, acute promyelocytic leukemia, acute monocytic leukemia, acute myelomonocytic leukemia; malignant lymphoma such as Burkitt's and Non-Hodgkin's; Lymphocytic leukemia such as acute lymphoblastic leukemia and chronic lymphoblastic leukemia; meninigioma; adenomas; adenocarcinomas such as small cell lung, kidney, uterous, prostate, bladder, ovary, colon; sarcomas such as liposarcoma, myxoid, synovial sarcoma, Ewing's tumor, alveolar, Wilm's tumor, neuroblastoma, testicular dysgerminoma, retinoblastoma, and melanoma.

Breast cancer as used herein means any tumor from any type of tissue found in the breast. Ovarian cancer as used herein means any tumor from any type of tissue found in the ovary. As defined herein "tumor antigen associated antibody" means antibodies directed at tumor antigens or tumor associated antigens.

In one embodiment the specific antibodies producing cells are detected. The culture of step b) may result in a supernatant, and the supernatant may be exposed to a tumor antigen, thereby allowing an antigen-antibody immune complex to form. Also, leukocytes or cellular fraction of the culture may be used and antibodies producing cells detecting expanded, cloned and immortalized.

In one embodiment, tumor antigens include but are not limited to: MUC-1, HERneu2, BrCa1, p53, Her-2-c-neu (AcIISAVVGIL-NH2), Carcinomembrionic antigen, prostate specific antigen, TAG-72, CA15-3, CA549, BA46, mammary serum antigen, mucinous carcinoma antigen, PEM peptide antigen, MUC1 glycosylation site peptide, MUC1 glycosylation site peptide glycosylated, MUC1 core tandem repeat peptide, MUC2 core tandem repeat peptide, MUC3 core tandem repeat peptide, MUC5AC peptide, SIMA, Lewis X antigen, Sialyl Lewis X antigen (BSA conjugate), I antigen(Gal1,4, GlcNAc, 1,3 Gal-R), T antigen, TN antigen (OSM) Sialyl Tn antigen. Antibodies can be looked at for either against "naked" antigens or against the complexes they form with products of transforming genes.

Tumor antigens that are needed for the detection of tumor specific antibodies can be in several forms: a) the whole cell (of a tumor), can serve as the antigen; b) cellular membranes the product of which can be used as antigens; c) the sequence of unique tumor structure or proteins (or segments of it) can be inserted via vectors to other producing entities (cells, bacteria, yeast, phage), and then individual proteins and structures will be produced; d) if the amino acid sequences of the tumor specific proteins are known, peptide that are fragments of the whole protein (or the whole protein itself) can be biochemically synthesized and used and antigen; and e) isolated from serum or other body fluid with high titre .

Most induced or transplanted experimental animal tumors immunize recipients against subsequent challenge with the same tumor but not against transplantation of normal tissues or other tumors. TAAs are particularly well demonstrated by chemical carcinogen-induced tumors, which tend to have individually specific antigens that vary from tumor to tumor, even with tumors induced by the same carcinogen; and by viral-induced tumors, which tend to show cross-reactivity between tumors induced by a given virus. Viral infections may result in "modified self" ; ie, new antigens recognized along with or in the context of the major histocompatibility complex.

Tumor antigens are formed by: (1) new genetic information introduced by a virus; (2) alteration of genetic function by carcinogens, possibly through activation of a proto-oncogene, by which genetic material that is normally inactive (except perhaps during embryonic development) is activated to an oncogene and becomes expressed in the cell phenotype; (3) uncovering the antigens that are normally present on normal cells or "buried" in the cell membrane, through the inability of neoplastic cells to synthesize membrane constituents (eg, sialic acid); and (4) release of antigens that are normally sequestered in the cell or its organelles, through the death of neoplastic cells.

Techniques to demonstrate TAAs in animal tumors include standard tissue transplantation methods, immunofluorescence, cytotoxicity tests using dye uptake or radioisotope release, prevention of tumor growth in vitro or in vivo by exposing the tumor to lymphoid cells or sera from immunized donors, delayed hypersensitivity skin tests, and lymphocyte transformation in vitro.

Evidence for TAAs in human malignant tumors has been shown with several neoplasms, including Burkitt's lymphoma, neuroblastoma, malignant melanoma, osteosarcoma, and some GI carcinomas. Choriocarcinomas in women possess paternally derived histocompatibility antigens that may serve as "tumor-specific" antigens in eliciting an immune response. The complete cure of choriocarcinomas by chemotherapy may be attributable, at least in part, to such an immune response. Unfortunately, although they may possess TAAs, apparently not all human tumors are antigenic in the host.

Thus any structure that appears to the immune system as new can be considered as a tumor antigen. Another general example of a normal protein appearing and serving as a new antigen for the immune system is that of normal proteins appearing in new context, such as embryonic proteins on mature (or adult) cells. Then the new epitopes would be in the interface between the two structures, an interface that forms new forms and structures for the immune system to see and react to.

Tumor-Associated Antigen (TAAs) are antigens associated with tumor cells that are also present on normal cells but in less amount or at different phase of a subjects life, i.e. embryonic proteins.

This invention provides a method of detecting a subject with a tumor , comprising the following steps: a) obtaining a whole blood sample from the subject; b) incubating the whole blood sample in a culture in the presence of a media containing a mitogen with or without antigen, so as to induce polyclonal and specific activation of lymphocytic cells leading to antibody production; c) treating the cells so as to recover nucleic acid sequences; d) contacting the resulting nucleic acid sequences with single-stranded labeled oligonucleotide primers, the primers being capable of specifically hybridizing with the nucleic acid sequence of the antibody produced as a result of exposure to an tumor antigen , under hybridizing conditions; and e) detecting the presence of the amplification product, the presence thereof being indicative of the subject having cancer.

The cells of step c) may be treated so as to expose nucleic acid sequences of the cell. Methods of exposure are known to those skilled in the art. Alternatively the resulting nucleic acid sequences of step d) may be amplified by a pair of primers which hybridizes to the nucleic acid sequence so as to obtain a double-stranded amplification product. Amplification product may be detected.

In another embodiment primers contact the resulting nucleic acid sequences capable of hybridizing with nucleic acid sequence at the edge of the variable region, under hybridizing conditions and specifically detected. Primers and probes of tumor antigens and tumor associated antibodies are known to those skilled in the art.

This invention provides a method of detecting a subject with a tumor, comprising the following steps: a) obtaining a whole blood sample from the subject; b) incubating the whole blood sample in a culture in the presence of a media

containing a mitogen with or without antigen, so as to induce polyclonal and specific activation of lymphocytic cells leading to antibody production; c) treating the cells so as to separately recover nucleic acid sequences; d) contacting the resulting nucleic acid sequences with multiple pairs of single-stranded labeled oligonucleotide primers, each such pair being capable of specifically hybridizing with the nucleic acid sequence of the antibody produced as a result of exposure to a tumor antigen , under hybridizing conditions; e) amplifying any nucleic acid sequences to which a pair of primers hybridizes so as to obtain a double-stranded amplification product; f) treating any such double-stranded amplification product so as to obtain single-stranded nucleic acid sequences therefrom; g) contacting any resulting single-stranded nucleic acid sequences with a labeled oligonucleotide probes, being capable of specifically hybridizing with such the tumor antigen associated antibodies antibody, under hybridizing conditions; h) contacting any resulting hybrids with a detectably marked tag which is capable of specifically forming a complex with the labeled-probe, when the probe is present in such a complex, under complexing conditions; and i) detecting the presence of any resulting complexes, the presence thereof being indicative of the subject having cancer.

This invention provides for the collection of the interface layer, also known as the buffy coat (with varying volumes of red cells and plasma) prior to incubation with the mitogen with or without antigen, thereby putting in culture a part of the blood-sample-volume that is enriched in leukocytes. Methods of leukocyte enrichment are known to those skilled in the art. For example, one may directly suction of the buffy coat as the middle layer in the tube or remove first most of the plasma volume and then take the buffy-coat with the remaining plasma. In the alternative, one may lyse the red cells, centrifuge and collect the leukocytes from the bottom.

In one embodiment the method comprises the following steps: a) obtaining a whole blood sample from the subject; b) allowing the whole blood sample to separate into a plurality of component layers including an interface layer; c)

collecting the interface layer from the whole blood sample d) incubating the interface layer in a culture in the presence of a mitogen with or without antigen containing media, so as to induce polyclonal and specific activation of lymphocytic cells leading to antibody production; e) exposing the resultant culture of step d) to an antigen, thereby allowing an antigen-antibody immune complex to form; f) detecting the antigen-antibody immune complex of step; e) wherein the presence of virus specific antibodies is indicative of the subject being exposed to the antigen.

In one embodiment the method comprises the following steps: a) obtaining a whole blood sample from the subject; b) allowing the whole blood sample to separate into a plurality of component layers including an interface layer; c) collecting the interface layer from the whole blood sample; d) incubating the interface layer in a culture in the presence of a mitogen with or without antigen containing media, as to induce polyclonal and specific activation of B lymphocytic cells and viral replication; e) treating the cells so as to separately recover nucleic sequences; f) contacting the resulting nucleic acid sequences with multiple Pairs of single-stranded labeled oligonucleotide primers, each such pair being capable of specifically hybridizing with the human immunodeficiency virus, under hybridizing conditions; g) amplifying any nucleic acid sequences to which a pari of primers hybridizes so as to obtain a double-stranded amplification product; h) treating any such double-stranded amplification product so as to obtain single-stranded amplification product so as to obtain single-stranded nucleic acid sequences with a labeled oligonucleotide probes, being capable of specifically hybridizing with the human immunodeficiency virus, under hybridizing conditions; j) contacting any resulting hybrids with a detectably marked tag which is capable of specifically forming a complex with the labeled-probe, when the probe is present in such a complex, under complexing conditions; and k) detecting the presence of any resulting complexes, the presence thereof being indicative of the presence of the human immunodeficiency virus.

This invention provides a method for the detection of tumor antigen associated antibodies , comprising: a) obtaining a whole blood sample from the subject; b) incubating the whole blood sample in a culture in the presence of a media containing a mitogen with or without antigen, so as to induce polyclonal and specific activation of lymphocytic cells leading to antibody production; c) exposing the cells so as to separately recover nucleic acid sequences of the tumor antigen associated antibody ; d) performing reverse transcription of an RNA of a tumor antigen associated antibodies in a sample to produce a DNA copy; e) performing polymerase chain reaction amplification of the DNA to produce a plurality of DNA copies; f) performing hybridization of the DNA copies to a plurality of complementary DNA probes to detect the DNA content of the sample; thereby detecting tumor antigen associated antibodies .

This invention provides a method for amplifying a target RNA sequence in a sample, comprising: a) obtaining a whole blood sample from the subject; b) incubating the whole blood sample in a culture in the presence of a media containing a mitogen with or without antigen, so as to induce polyclonal and specific activation of lymphocytic cells leading to antibody production; c) exposing the cells so as to separately recover nucleic acid sequences of the tumor antigen associated antibody; d) treating the sample in a reaction mixture comprising a first and second primer, wherein the first primer is sufficiently complementary to the target RNA to hybridize therewith and initiate synthesis of a cDNA sequence complementary to the target RNA, and the second primer is sufficiently homologous to the target RNA to hybridize to the cDNA and initiate synthesis of an extension product, and a thermostable DNA polymerase in the presence of all four deoxyribonucleoside triphosphates, in an appropriate buffer, wherein the buffer comprises Mn+2 , at a temperature sufficient for the thermostable DNA polymerase to initiate synthesis of an extension product of the first primer to provide a cDNA sequence complementary to the target RNA; e) treating the reaction mixture at an appropriate temperature to provide single-stranded cDNA; f) treating the reaction mixture at an appropriate temperature for the thermostable DNA polymerase to initiate synthesis of an

extension product of the second primer to provide a double-stranded cDNA sequence; and g) amplifying the double-stranded cDNA sequence of step e) by a polymerase chain reaction. In a preferred embodiment, the buffer comprises manganese acetate (also written Mn(OAc)2 or Mn(CH3CO2)2), Bicine-KOH (Bicine is N,N-Bis(2-Hydroxyethyl)glycine), and potassium acetate (also written KOAc or KCH3CO2).

The present invention also contemplates labeling methods wherein the oligonucleotide probe sequences have at least one label attached or integrated into its structure. Labels are generally intended to facilitate the detection of the tumor antigen. Labels are chosen from the group consisting of enzymes, fluorophores, high-affinity conjugates, chemiphores and radioactive atoms ("radiolabels"). While other labels may be used, the present invention contemplates: 1) the enzymes alkaline phosphatase, beta -galactosidase and glucose oxidase; 2) the affinity conjugate system of biotin-avidin; 3) the fluorophore that is fluorescein; 4) the chemiphore that is luminol; and 5) the preferred radiolabels 3H, 14C and 32P.

This invention provides a method of detecting a subject with a tumor , comprising the following steps: a) obtaining a whole blood sample from the subject; b) incubating the whole blood sample in a culture in the presence of a media containing a mitogen with or without antigen,so as to induce polyclonal and specific activation of lymphocytic cells leading to antibody production; c) exposing the resultant culture of step b) to an specific tumor antigen associated antibody, thereby allowing an antibody- tumor antigen immune complex to form; d) detecting the antibody- tumor antigen immune complex of step c); wherein the presence of tumor antigen associated antibodies is indicative of the subject having ovarian or breast cancer.

In one embodiment, the culture of step b) results in a supernatant, and the supernatant is exposed to a specific tumor antigen associated antibody, thereby allowing an antibody- tumor antigen immune complex to form.

This invention provides a kit for the detection of specific tumor antigen associated antibodies from a subject, comprising: a container for collecting whole blood samples, wherein the container contains a media containing mitogen with or without antigen, effective to induce polyclonal and specific activation of lymphocytic cells leading to antibody production and an assay for the detection of the specific tumor antigen associated antibody. It is contemplated that there may be multiple detection assays for a number of different antibodies which bind to a multitute of antigens that are bound to the container containing the media.

In one embodiment, a sample of leucocytic cells is obtained from the subject and incubated in a culture in the presence of a media containing a mitogen with or without antigen, so as to induce polyclonal and specific activation of lymphocytic cells. In another embodiment a sample of lymphocytic cells is obtained from the subject and incubated in a culture in the presence of a media containing a mitogen with or without antigen, so as to induce polyclonal and specific activation of B lymphocytic cells. In another embodiment a sample of PBMC is obtained from the subject and incubated in a culture in the presence of a media containing a mitogen with or without antigen, so as to induce polyclonal and specific activation of B lymphocytic cells. Single or multiple antigens may be bound to the container.

In accordance with the present invention, a blood sample is drawn into a test tube, such as a vacutube, containing an effective concentration of a solution of a mitogen with or without antigen, such as pokeweed mitogen and relevant tumor antigen and peptide. The blood sample to be tested is cultured *in vitro* in the presence of the pokeweed mitogen. Other activators of B lymphocytic cells or the humoral system which leads to antibody production or secretion and activators of the Th_2 type immune response may be used in place of or in addition to the pokeweed mitogen to achieve the same function. After incubation, an aliquot is taken from the fluid and is then assayed for the presence of desired tumor antigen associated antibodies using standard ELISA

procedures and/or Western Blot analysis. If the sample is to be assayed at a later date, the blood may be centrifuged and the supernatant fluid may be collected, frozen and stored. Results may be verified utilizing the technique of polymerase chain reaction (PCR)/FACS. The cellular fractions in the container may be used in PCR or ELISPOT. Other methods for detecting antibodies are known to those skilled in the art.

The method of the present invention includes optionally separating the blood cells from the fluid portion of the blood so that the presence of tumor antigen associated antibodies can be determined. The separation of the blood cells from the fluid portion of the blood can be done by any of several methods well known to those of ordinary skill in the art, including centrifugation, filtration or density gradient. It is to be understood, that the blood cells do not need to be physically separated from the fluid. After incubation of the whole blood with the mitogen with or without antigen, fluid from the blood can easily be extracted and tested for tumor antigen associated antibody. Optionally, the red blood cells can be lysed either by mild osmotic shock or with a mild detergent. In this way, the white blood cells remain viable.

In one embodiment, of the present invention, whole blood is collected in a blood collection tube containing culture medium and mitogen with or without antigen. The blood samples are then incubated with an approximately 1:500 final dilution of pokeweed mitogen at a concentration of $2x10^6$ viable lymphocytes per ml for 1-12 days at 37°C in a 7% CO_2 humidified atmosphere. The blood may be centrifuged and the supernatant fluid is collected immediately and assayed within approximately 1-12 hours or frozen later for testing for reactive specific antibodies by ELISA/ELISPOT and/or Western blot techniques. In the alternative, an aliquot of fluid or cells or cellular components may be taken directly from the sample.

The present invention also includes a kit comprising a blood collection container containing an effective concentration of mitogen with or without antigen therein.

The container can optionally contain a culture medium. The preferred container is a test tube. The blood collection container can be plastic, glass, or any other material that is compatible with culturing blood. It is to be understood that the present invention also includes blood containing means other then a blood collection tube including, but not limited to, microtiter plates containing wells in which the blood can be incubated, tissue culture flasks, glass flasks such as an Erlenmeyer flask, and any other container in which the blood can be cultured.

Tumor antigen associated antibody which specifically bind specific tumor antigen are known to those skilled in the art. Further, antibodies may be used as tags to bind to the tumor antigen. In one embodiment, the antibody is a monoclonal antibody. In another embodiment the antibody is a polyclonal antibody.

This invention provides an isolated nucleic acid molecule encoding a peptide having the amino acid sequence SGSGHGVTSAPDTR (SEQ. ID. No. 1). This invention provides an isolated nucleic acid molecule encoding a peptide having the amino acid sequence SGSGAPDTRPAPGSTAP (SEQ. ID. No. 2). This invention provides an isolated nucleic acid molecule encoding a peptide having the amino acid sequence IISAVVGIL (SEQ. ID. No. 3). This invention provides a tag on the nucleic acids for binding to surfaces.

This invention provides a nucleic acid sequence of at least 15 nucleotides capable of specifically hybridizing with a sequence of the nucleic acid sequence of SEQ. ID NOs. 1,2, or 3.

This invention provides an antibody directed to peptide SGSGHGVTSAPDTR (SEQ ID NO. 1), SGSGAPDTRPAPGSTAP, (SEQ ID NO. 2), or IISAVVGIL (SEQ ID NO. 3). In one embodiment, the antibody may a monoclonal or polyclonal. The antibody may be tagged. In another embodiment this invention provides a ligand which binds to the peptide.

This invention also provides a method of imaging ovarian or breast cancer in a subject which comprises administering to the subject at least one antibody directed against the peptide of SEQ. ID. NO. 1, 2, or 3, capable of binding to the cell surface of the cancer cell and labeled with an imaging agent under conditions so as to form a complex between the monoclonal antibody and the cell surface antigen. This invention further provides a composition comprising an effective imaging amount of the antibody directed against the peptide and a pharmaceutically acceptable carrier. Knowledge of the antibodies to the peptide sequence is useful for treatment, i.e.immunotoxin therapy or diagnostic either in vitro, i.e. immunohistochmistry or in-vivo diagnostic use, i.e. radiolabelling for targeting with toxins.

The kit can detect the presence of an tumor antigen associated antibody specifically reactive with a tumor antigen or an immunoreactive fragment thereof. The kit can include an tumor antigen bound to a substrate, a secondary tumor antigen reactive with the antibody and a reagent for detecting a reaction of the secondary tumor antigen with the antibody. Such a kit can be an antibody capture assay kit, such as an ELISA kit, and can comprise the substrate, primary and secondary antibodies when appropriate, and any other necessary reagents such as detectable moieties, enzyme substrates and color reagents as described above. The antibody capture diagnostic kit can, alternatively, be an immunoblot kit generally comprising the components and reagents described herein. The particular reagents and other components included in the diagnostic kits of the present invention can be selected from those available in the art in accord with the specific diagnostic method practiced in the kit. Such kits can be used to detect the antibody in biological samples, such as tissue and bodily fluid before and after culture obtained from a subject.

This invention provides a kit for the detection of specific tumor antigen associated antibody from a subject, comprising: a vacuum sealed container for collecting whole blood samples, wherein the container contains a media containing mitogen with or without antigen, effective to induce polyclonal and

specific activation of lymphocytic cells leading to antibody production and an assay for the detection of one or more tumor associated antibodies.

In the present invention, detecting the reaction of the ligand with the tumor antigen can be further aided, in appropriate instances, by the use of a secondary antibody or other ligand which is reactive, either specifically with a different epitope or nonspecifically with the ligand or reacted antibody.

This invention provides a method of diagnosing a subject with cancer which comprises: a) obtaining a whole blood sample from the subject; b) incubating the whole blood sample in a culture in the presence of a media containing a mitogen with or without antigen, to induce polyclonal and specific activation of lymphocytes; c) obtaining a nucleic acid sequence from lymphocytes; d) contacting the nucleic acid sequence with a labeled nucleic acid sequence of at least 15 nucleotides capable of specifically hybridizing with the isolated DNA, under hybridizing conditions; and e) determining the presence of the nucleic acid sequence hybridized, the presence of which is indicative of tumor antigen associated antibodies in the subject, thereby diagnosing the subject.

In one embodiment, the DNA sequence from the tumor antigen associated antibodies is amplified before step (b). In another embodiment PCR is employed to amplify the nucleic acid sequence. Methods of amplifying nucleic acid sequences are known to those skilled in the art.

Tumor antigen associated antibodies agent screening assays which determine whether or not a drug may used is contemplated in this invention. Such assays comprise incubating whole blood, lymphocytes, such as B cells, or PBMC with the mitogen with or without antigen and the tumor antigen associated antibodies drug and evaluate whether the cell expresses the tumor antigen associated antibodies and determining therefrom the effect of the compound on the activity of such agent.

The present invention relates to an improved assay for detecting a subject exposed to an antigen. This invention is contemplated for use as: 1) a diagnostic to determine if an individual has been exposed to an antigen, 2) a prognostic indicator, and 3) a method to monitor the effectiveness of anti-antigen treatment or the effectiveness of a new potential anti-antigen agent.

This invention provides an *in vitro* method for the detection of antibodies directed against an antigen in a sample obtained from a subject, comprising the following steps: a) obtaining a whole blood sample from the subject; b) incubating the whole blood sample in a culture in the presence of a mitogen with or without antigen containing media,so as to induce polyclonal and specific activation of lymphocytic cells leading to antibody production; c) exposing the resultant culture of step b) to an antigen, thereby allowing an antigen-antibody immune complex to form; d) detecting the antigen-antibody immune complex of step c); wherein the presence of specific antigen antibodies is indicative of the subject being exposed to an antigen.

In one embodiment, a sample of leucocytic cells is obtained from the subject and incubated in a culture in the presence of a media containing a mitogen with or without antigen, so as to induce polyclonal and specific activation of B lymphocytic cells. In another embodiment a sample of lymphocytic cells is obtained from the subject and incubated in a culture in the presence of a media containing a mitogen with or without antigen, so as to induce polyclonal and specific activation of B lymphocytic cells. In another embodiment a sample of PBMC is obtained from the subject and incubated in a culture in the presence of a media containing a mitogen with or without antigen, so as to induce polyclonal and specific activation of B lymphocytic cells.

In one embodiment, the specific antibodies producing cells are detected. In another embodiment, the culture of step b) results in a supernatant, and the supernatant is exposed to an antigen, thereby allowing an antigen-antibody

immune complex to form. In another embodiment the cellular fraction of the culture is used. In another embodiment, leukocytes are used.

This invention further provides a method of detecting a subject exposed to an antigen, comprising the following steps: a) obtaining a whole blood sample from the subject; b) incubating the whole blood sample in a culture in the presence of a media containing a mitogen with or without antigen, so as to induce polyclonal and specific activation of lymphocytic cells leading to antibody production; c) treating the cells so as to recover nucleic acid sequences; d) contacting the resulting nucleic acid sequences with single-stranded labeled oligonucleotide primers, the primers being capable of specifically hybridizing with the nucleic acid sequence of the antibody produced as a result of exposure to an antigen, under hybridizing conditions; and e) detecting the presence of the amplification product, the presence thereof being indicative of the presence of the antigen.

The cells of step c) may be treated so as to expose nucleic acid sequences of the cell. Methods of exposure are known to those skilled in the art. Alternatively, the resulting nucleic acid sequences of step d) are amplified by a pair of primers which hybridizes to the nucleic acid molecule so as to obtain a double-stranded amplification product. The amplification product may be detected as well.

In another embodiment, primers contact the resulting nucleic acid sequences capable of hybridizing with nucleic acid sequence at the edge of the variable region, under hybridizing conditions and specifically detected.

This invention also provides a method of detecting a subject exposed to an antigen, comprising the following steps: a) obtaining a whole blood sample from the subject; b) incubating the whole blood sample in a culture in the presence of a media containing a mitogen with or without antigen, so as to induce polyclonal and specific activation of lymphocytic cells leading to antibody production; c) treating the cells so as to separately recover nucleic acid

sequences; d) contacting the resulting nucleic acid sequences with multiple pairs of single-stranded labeled oligonucleotide primers, each such pair being capable of specifically hybridizing with the nucleic acid sequence, under hybridizing conditions; e) amplifying any nucleic acid sequences to which a pair of primers hybridizes so as to obtain a double-stranded amplification product; f) treating any such double-stranded amplification product so as to obtain single-stranded nucleic acid sequences therefrom; g) contacting any resulting single-stranded nucleic acid sequences with a labeled oligonucleotide probes, being capable of specifically hybridizing with the nucleic acid sequence of the antibody produced as a result of exposure to an antigen, under hybridizing conditions; h) contacting any resulting hybrids with a detectably marked tag which is capable of specifically forming a complex with the labeled-probe, when the probe is present in such a complex, under complexing conditions; and i) detecting the presence of any resulting complexes, the presence thereof being indicative of the presence of the antigen .

"Antigens" include but are not limited to: immunogens, allergens, carcinogens, alloantigens, self-antigens(auto-anitgens), cancer antigens, cancer associated antigens, transplantation antigens, blood group antigens, or pollutants. As defined herein all antigens elicit an immune response. Thus looking for an antibody (or antibodies) against it means that you would use the whole or segments (peptides) of it as the antigen for the detection systems and tests.

The antigen may be proteins, peptides, fragments, disassociated, peptides (of any length); lipoproteins; carbohydrates, such as glycoproteins and glycolipids (both have representatives on the bacteria walls, blood group antigens, and myeloma structures for example); oligosaccharides/ polysaccharides; lipids/fats; haptens; and chemicals such as TNP (trinitrophenyl), benzene arsenate, and non-organic compounds.

Helicobacterium pylori, the bacteria that has been implicated as the cause of a large part of the peptic ulcers. This bacteria has many antigenic/antigenic

epitopes or structures. The entire bacteria, or some of its proteins, carbohydrates, mucins, or peptides may serve as an antigen for the antibody detection.

This invention also provides a method for amplifying a target RNA sequence in a sample, comprising: a) obtaining a whole blood sample from the subject; b) incubating the whole blood sample in a culture in the presence of a media containing a mitogen with or without antigen, so as to induce polyclonal and specific activation of lymphocytic cells leading to antibody production; c) treating the cells so as to separately recover nucleic acid sequences of the antibody resulting from exposure to the antigen; d) treating the sample in a reaction mixture comprising a first and second primer, wherein the first primer is sufficiently complementary to the target RNA to hybridize therewith and initiate synthesis of a cDNA sequence complementary to the target RNA, and the second primer is sufficiently homologous to the target RNA to hybridize to the cDNA and initiate synthesis of an extension product, and a thermostable DNA polymerase in the presence of all four deoxyribonucleoside triphosphates, in an appropriate buffer, wherein the buffer comprises Mn+2 , at a temperature sufficient for the thermostable DNA polymerase to initiate synthesis of an extension product of the first primer to provide a cDNA sequence complementary to the target RNA; e) treating the reaction mixture at an appropriate temperature to provide single-stranded cDNA; f) treating the reaction mixture at an appropriate temperature for the thermostable DNA polymerase to initiate synthesis of an extension product of the second primer to provide a double-stranded cDNA sequence; and g) amplifying the double-stranded cDNA sequence of step e) by a polymerase chain reaction. In a preferred embodiment, the buffer comprises manganese acetate (also written Mn(OAc)2 or Mn(CH3CO2)2), Bicine-KOH (Bicine is N,N-Bis(2-Hydroxyethyl)glycine), and potassium acetate (also written KOAc or KCH3CO2).

This invention provides a kit for the detection of specific antibodies resulting from exposure to an antigen from a subject, comprising: a container for collecting whole blood samples, wherein the container contains a media

containing mitogen with or without antigen, effective to induce polyclonal and specific activation of lymphocytic cells leading to antibody production and an assay for the detection of the specific specific antibodies resulting from exposure to an antigen. The container may be a vacuum sealed container for collecting whole blood samples, and contains a media containing mitogen with or without antigen, effective to induce polyclonal and specific activation of lymphocytic cells leading to antibody production. Single or multiple antigens may be bound to the container.

In accordance with the present invention, a blood sample is drawn into a test tube, such as a vacutube, containing an effective concentration of a solution of a mitogen with or without antigen, such as pokeweed mitogen. The blood sample to be tested is cultured *in vitro* in the presence of the pokeweed mitogen. Other activators of B lymphocytic cells or the humoral system which leads to antibody production or secretion, that activate a Th_2 type immune response may be used in place of or in addition to the pokeweed mitogen to achieve the same function. After incubation, an aliquot is taken from the fluid and is then assayed for the presence of desired antigen using standard ELISA procedures and/or Western Blot analysis. If the sample is to be assayed at a later date, the blood may be centrifuged and the supernatant fluid may be collected, frozen and stored. Results may be obtained utilizing any other technique of detection. Cellular fractions may be used with PCR and ELISPOT.

The method of the present invention includes optionally separating the blood cells from the fluid portion of the blood so that the presence of antibody can be determined. The separation of the blood cells from the fluid portion of the blood can be done by any of several methods well known to those of ordinary skill in the art, including centrifugation, filtration or density gradient. It is to be understood, that the blood cells do not need to be physically separated from the fluid. After incubation of the whole blood with the mitogen with or without antigen, fluid from the blood can easily be extracted and tested for antibody.

Optionally, the red blood cells can be lysed either by mild osmotic shock or with a mild detergent. In this way, the white blood cells remain viable.

Antigens or antigens which specifically bind specific antibodies of the to antigen are known to those skilled in the art. Further, antibodies may be used as tags to bind to the antigen. In one embodiment the antibody is a monoclonal antibody. In another embodiment the antibody is a polyclonal antibody.

Another method of immunoenzymatic detection of the presence of specific antibodies directed against one or more of the antigens is the Western blot. The antigens are separated electrophoretically and transferred to a nitrocellulose membrane or other suitable support. The body fluid to be tested is then brought into contact with the membrane and the presence of the immune complexes formed is detected by the method already described. In a variation on this methods, purified antigen is applied in lines or spots on a membrane and allowed to bind. The membrane is subsequently brought into contact with the body fluid before and after culture to be tested and the immune complexes formed are detected using the previously described techniques.

This invention provides a method of diagnosing a subject exposed to an antigen which comprises: a) obtaining a whole blood sample from the subject; b) incubating the whole blood sample in a culture in the presence of a media containing a mitogen with or without antigen, to induce polyclonal and specific activation of lymphocytes cells; c) obtaining a nucleic acid sequence from lymphocytes; d) contacting the nucleic acid sequence with a labeled nucleic acid sequence of at least 15 nucleotides capable of specifically hybridizing with the isolated DNA, under hybridizing conditions; and e) determining the presence of the nucleic acid sequence hybridized, the presence of which is indicative of exposure to an antigen in the subject, thereby diagnosing the subject.

In the above described methods, a size fractionation may be employed which is effected by a polyacrylamide gel. In one embodiment, the size fractionation is effected by an agarose gel. Further, transferring the DNA fragments into a solid matrix may be employed before a hybridization step. One example of such solid matrix is nitrocellulose paper.

A method for detecting the specific antibodies produced as a result of infection or exposure of antigen is the use of PCR and/or dot blot hybridization. The presence or absence of an antigen agent for detection or prognosis, or risk assessment for antigen includes Southern transfers, solution hybridization or non-radioactive detection systems, all of which are well known to those of skill in the art. Hybridization is carried out using probes. Visualization of the hybridized portions allows the qualitative determination of the presence or absence of the causal agent.

Antigen agent screening assays which determine whether or not a drug may used is contemplated in this invention. Such assays comprise incubating whole blood, lymphocytes, such as B cells, or PBMC with the mitogen with or without antigen and the antigen drug and evaluate whether the cell expresses the antigen and determining therefrom the effect of the compound on the activity of such agent.

The present invention relates to an improved assay for detecting a subject infected with virus. This invention is contemplated for use as: 1) a diagnostic to determine if an individual has been infected with virus, 2) a prognostic indicator, and 3)a method to monitor the effectiveness of anti-viral treatment (or the effectiveness of a new potential anti-viral drug or agent.

This invention provides an *in vitro* method for the detection of antibodies directed against a virus in a sample obtained from a subject, comprising the following steps: a) obtaining a whole blood sample from the subject; b) incubating the whole blood sample in a culture in the presence of a mitogen with or without antigen containing media,so as to induce polyclonal and specific

activation of lymphocytic cells leading to antibody production; c) exposing the resultant culture of step b) to an virus antigen, thereby allowing an antigen-antibody immune complex to form; d) detecting the antigen-antibody immune complex of step c); wherein the presence of virus specific antibodies is indicative of the subject being exposed to virus.

In one embodiment the specific antibodies producing cells are detected. The culture of step b) may result in a supernatant, and the supernatant may exposed to a virus antigen, thereby allowing an antigen-antibody immune complex to form. Also, leukocytes or cellular fraction of the culture may be used.

This invention provides a method of detecting a subject infected with a virus, comprising the following steps: a) obtaining a whole blood sample from the subject; b) incubating the whole blood sample in a culture in the presence of a media containing a mitogen with or without antigen, so as to induce polyclonal and specific activation of lymphocytic cells leading to antibody production; c) treating the cells so as to recover nucleic acid sequences; d) contacting the resulting nucleic acid sequences with single-stranded labeled oligonucleotide primers, the primers being capable of specifically hybridizing with the nucleic acid sequence the antibody produced as a result of exposure to virus , under hybridizing conditions; and e) detecting the presence of the amplification product, the presence thereof being indicative of the presence of the virus .

The cells of step c) may be treated so as to expose nucleic acid sequences of the cell. Methods of exposure are known to those skilled in the art. Alternatively the resulting nucleic acid sequences of step d) may be amplified by a pair of primers which hybridizes to the nucleic acid sequence so as to obtain a double-stranded amplification product. Amplification product may be detected.

In another embodiment primers contact the resulting nucleic acid sequences capable of hybridizing with nucleic acid sequence at the edge of the variable region, under hybridizing conditions and specifically detected.

This invention provides a method of detecting a subject infected with a virus, comprising the following steps: a) obtaining a whole blood sample from the subject; b) incubating the whole blood sample in a culture in the presence of a media containing a mitogen with or without antigen, so as to induce polyclonal and specific activation of lymphocytic cells leading to antibody production; c) treating the cells so as to separately recover nucleic acid sequences; d) contacting the resulting nucleic acid sequences with multiple pairs of single-stranded labeled oligonucleotide primers, each such pair being capable of specifically hybridizing with the nucleic acid sequence of the antibody produced as a result of exposure to virus, under hybridizing conditions; e) amplifying any nucleic acid sequences to which a pair of primers hybridizes so as to obtain a double-stranded amplification product; f) treating any such double-stranded amplification product so as to obtain single-stranded nucleic acid sequences therefrom; g) contacting any resulting single-stranded nucleic acid sequences with a labeled oligonucleotide probes, being capable of specifically hybridizing with such the virus antibody, under hybridizing conditions; h) contacting any resulting hybrids with a detectably marked tag which is capable of specifically forming a complex with the labeled-probe, when the probe is present in such a complex, under complexing conditions; and i) detecting the presence of any resulting complexes, the presence thereof being indicative of the presence of the virus .

Viral antigens that are needed for the detection of virus specific antibodies can be in several forms: a) whole, or inactivated virus which may be dissociated (by sonication, homogenization, detergent, enzymes, and the product can be used as antigens as a group or individually); c) the viral sequence (or segments of it) may be inserted via vectors to other producing entities (cells, bacteria, yeast phage), and then individual proteins and structures will be produced; d) peptide fragments of the whole protein (both naked and glycosylated or connected with lipids, Specific peptides either alone or tied/bound/connected/part of/expressed on larger or different particles/bodies/cells/carriers/surfaces) can be biochemically synthesized and used and antigen; and e) the interface between the virus and the

cell or the exact way the viral antigens are expressed on the surface of the infected cell could serve as a specific antigenic epitopes too.

Viruses include but are not limited to: avian encephalomyelitis virus, avian reovirus, avian paramyxovirus, avian influenza virus, avian adenovirus, fowl pox virus, avian coronavirus, avian rotavirus, chick anemia virus (agent), *Salmonella spp. E. coli, Pasteurella spp., Bordetella spp., Eimeria spp., Histomonas spp., Trichomonas spp.*, avian encephalomyelitis virus, avian reovirus, avian paramyxovirus, avian influenza virus, avian adenovirus, fowl pox virus, avian coronavirus, avian rotavirus, chick anemia virus (agent), *Salmonella spp. E. coli, Pasteurella spp., Bordetella spp., Eimeria spp., Histomonas spp., Trichomonas spp.*, Poultry nematodes, cestodes, trematodes, poultry mites/lice, poultry protozoa.

Viruses of nonhuman primates are included but not limited to: Aotine herpesvirus 1, Aotine herpesvirus 3, Cercopithecine herpesvirus 1 (B virus, HV simiae), Cercopithecine herpesvirus 2 (SA8), Cercopithecine herpesvirus 3 (SA6), Cercopithecine herpesvirus 4 (SA15), Cercopithecine herpesvirus 5 (African green monkey cytomegalovirus), Cercopithecine herpesvirus 6 (Liverpool vervet monkey virus), Cercopithecine herpesvirus 7 (P a t a s monkey HV; MMV or PHV delta HV), Cercopithecine herpesvirus 8 (Rhesus monkey cytomegalovirus), Cercopithecine herpesvirus 9 (Medical Lake macaque HV simian varicella HV) , Cercopithecine herpesvirus 10 (Rhesus leukocyte assoc. HV strain 1), Cercopithecine herpesvirus 12 (HV papio, baboon HV), Cercopithecine herpesvirus 13 (Herpesvirus cyclopis), Cercopithecine herpesvirus 14 (African green monkey EBV-like virus), Cercopithecine herpesvirus 15 (Rhesus EBV-like HV) , Ateline herpesvirus 1 (Spider monkey HV), Ateline herpesvirus 2 (HV ateles), Callitrichine herpesvirus (HV saguinus), Callitrichine herpesvirus (SSG, marmoset cytomegalovirus), Cebine herpesvirus 1 (Capuchin HV), Cebine herpesvirus 2 (Capuchin HV), Pongine herpesvirus 1 (Chimpanzee HV; pan HV), Pongine herpesvirus 2 (Orangutan HV), Pongine herpesvirus 3 (Gorilla HV), Saimiriine herpesvirus 1 (Marmoset

HV, herpes T, HV), tamarinus, HV platyrrhinae, (type Saimiriine herpesvirus 2) Squirrel monkey HV, and HV saimiri.

Viruses of mammals include but are not limited to: Bovine herpesvirus 1 - 5, Ovine herpesvirus 1-2, Alcelaphine herpesvirus 1, Parvovirus (including mice minute virus , Aleutian mink disease, bovine parvovirus, canine parvovirus, chicken parvovirus, feline panleukopenia, feline parvovirus, goose parvovirus, HB parvovirus, H-1 parvovirus, Kilham rat lapine parvovirus, mink enteritis) Erythrovirus (including adeno-associated type 1-5, bovine adeno-associated, canine adeno-associated, equine adeno-associated, ovine adeno-associated).

Viruses include but are not limited to: Cauliflower, Badnaviruses, Geminiviruses, Plant Reoviruses, Cryptoviruses, Rhabdoviridae, Tomato Spotted, Tenuiviruses, Tobacco, Potato Virus, Potyviridae, Closteroviruses, Turnip Yellow, Tomato Bushy, Luteoviruses, Sequiviridae, Tobacco, Cowpea, Tobacco, Pean Enation, Red Clover, Brome, Cucumber, Alfalfa, Barley,Beet Necrotic, dsRNA, Saccharomyces cerevisiae, Cryphonectria parasitica, Leishmania, Giardia Lamblia, Trichomonas, Chlorella, and Saccharomyces cerevisiae.

Further viruses from the following family are included: Baculoviridae and Nudiviruses, Polydnaviridae, Ascoviridae, Nodaviridae Tetraviridae, Tetraviridae, Tombusviridae, Coronaviridae, Flaviviridae, Togaviridae, Bromoviridae, Barnaviridae, Totiviridae, Partitiviridae, Hypoviridae, Paramyxoviridae, Rhabdoviridae, Filoviridae, Orthomyxoviridae, Bunyaviridae, Arenaviridae, Leviviridae, Picornaviridae, Sequiviridae, Comoviridae, Potyviridae, Calciviridae, Astroviridae, Nodaviridae, Inoviridae, Microviridae, Geminiviridae, Circoviridae, Parvoviridae, Haepadnaviridae, Retroviridae, Cystoviridae, Reoviridae, Birnaviridae, Myoviridae, Siphoviridae, Podoviridae, Tectiviridae, Corticoviridae, Plasmaviridae, Lipothrixviridae, Fuselloviridae, Poxviridae, African swine fever-like viruses, Iridoviridae, Phycodnaviridae, Baculoviridae, Herpesviridae, Adenoviridae, Papovaviridae, Polydnaviridae, Picornaviridae, Caliciviridae, Astroviridae, Togaviridae, Flaviviridae,

Coronaviridae, Arterivirus, Paramyxoviridae, Rhabdoviridae, Filoviridae, Orthomyxoviridae, Bunyaviridae, Arenaviridae, Reoviridae, Birnaviridae, Retroviridae, Hepadnaviridae, Circoviridae, Parvoviridae, Papovaviridae, Adenoviridae, Herpesviridae, Poxviridae, Iridoviridae,

Viruses include but are not limited to: Marek's disease virus (fowl), Mink Enteritis virus, Minute virus of mice, Mouse hepatitis viruses, Mouse mammary tumor virus, Mouse poliomyelitis virus (Theiler's virus) Mucosal disease virus (cattle), Myxoma virus, Nairobi sheep disease virus, Newcastle disease virus (fowl), orf virus (contagious pustular dermatitis virus), Parainfluenza virus 3, Parainfluenza virus 1 (Sendai virus), Peste-des-petits-ruminants virus (sheep and goats), Pneumonia virus of mice, Progressive penumonia virus of sheep, Psudocowpox virus (milker's nodule virus), Pseudorabies virus, Rabbit hemorrhagic disease virus, Rabies virus, Reoviruses 1-3, Rift Valley fever virus, Rinderpest virus, Rotaviruses of many species, Scrapie agent (sheep and goat), Sheeppox virus, Shope papillomavirus, Simian immunodeficiency viruses, Swine vesicular disease virus, Swinepox virus, Tick-borne encephalitis viruses, Transmissible gastroenteritis virus (swine), Turkey bluecomb virus, Venezuelan equine encephalitis virus, Vesicular exanthema virus (swine), Vesicular stomatitis virus, Wasting disease of deer and elk, Wesselsbron virus, Western equine encephalitis virus, African horsesickness viruses 1-9, African swine fever virus, Aleutian mink disease virus, Avian reticuloendotheliosis virus, Avian sarcoma and leukosis viruses, B virus (Cercopithecus herpesvirus), Berne virus (horses), Bluetongue viruses 1-25, Border disease virus (sheep), Borna disease virus (horses), Bovine enteroviruses 1-7, Bovine ephemeral fever virus, Bovine immunodeficiency virus, Bovine leukemia virus, Bovine mamillitis virus, Bovine papillomaviruses, Bovine papillomaviruses, Bovine papular stomatitis virus, Bovine respiratory syncytial virus, Bovine virus diarrhea virus, Breda virus (calves), Canine adenovirus 2, Canine distemper virus, Canine parvovirus, Caprine arthritis-encephalitis virus, Cowpox virus, Eastern equine encephalitis virus, Ebola virus, Ectromelia virus (mousepox virus), Encephalomyocarditis virus, Epizootic hemorrhagic disease viruses (deer), Equine abortion virus

(EHV1), Equine adenoviruses, Equine arteritis virus, Equine coital exanthema virus (EHV3), Equine infectious anemia virus, Equine rhinopneumonitis virus (EHV4), Feline calicivirus, Feline immunodeficiency virus, Feline infectious peritoniitis virus, Feline panleukopenia virus, Feline sarcoma and leukemia viruses, Fibroma viruses of rabbits and hares and squirrels, Foot-and-mouse disease viruses, Fowlpox virus, Hemagglutinating encephalomyelitis virus (swine), Hog cholera virus, Infectious bovine rhinotrachetitis virus, Infectious bronchitis virus (fowl), Infectious bursal disease virus (fowl), Infectious canine hepatitis virus, Infectious hematopoietic necrosis virus (fish), Infectious laryngotrachetis virus, infectious hematopoietic necrosis virus (fish), Influenza viruses of swine, horses, seals, and fowl, Japanese encephalitis virus, Lactic dehydrogenase virus (mice), Lymphocytic choriomeningitis virus, Maedi/visna virus (sheep), Marburg virus, Rocio virus, Ross River virus, Rubella virus, Russian spring-summer encephalitis virus, Sandfly fever-Naples virus, Sandfly fever-Sicilian virus, St. Louis encephalitis virus, SV 40 virus, Tahyna virus, Vaccinia virus, Varicella-zoster virus (human herpesvirus 3), Variola virus, Venezuelan equine encephalitis virus, Vesicular stomatitis viruses, West Nile virus, Eastern equine encephalitis virus, Yellow fever virus, Adenovirus 1-49, Astrovirus 1, 2, B virus (Cercopithecus herpesvirus), BK virus, Bunyamwera virus, California encephalitis virus, Central European encephalitis virus, Chikungunya virus, Colorado tick fever virus, Congo-Crimean hemorrhagic fever virus, Cowpox virus, Coxsacieviruses A 1-21 and A 24, Coxsackieviruses B 1-6, Creutzfeldt-Jakob disease agent, prions, Dengue viruses 1-4, Duvenhage virus, Eastern equine encephalitis virus, Ebola virus, Echoviruses 1-9 and 11-27 and 29-34, Enteroviruses 68-71, Epstein-Barr virus (human herpesvirus 4), Hantaan virus, Hepatitis A virus, Hepatitis B virus, Hepatitis C virus, Hepatitis delta virus, Hepatitis E virus, Herpes simplex viruses 1 and 2 (human herpesviruses 1 and 2), Human enteric coronavirus, Human enteric conoravirus, Human cytomeglovirus (human herpesvirus 5), Human herpesviruses 6A, 6B, and 7, Human immunodeficiency viruses 1 and 2 Human respiratory coronaviruses 229E and OC43, Human rotaviruses, Human T-lymphotropic viruses 1 and 2, Influenza viruses A and B, Japanese encephalitis virus, JC virus, Junin virus (Argentine

hemorrhagic fever virus), Kuru agent, Kyasanur forest virus, La Crosse virus, Lassa virus, Lymphocytic choriomeningitis virus, Macuopo virus (Bolivian hemorrhagic fever virus), Marburg virus, Mayaro virus, Measles virus, Mokola virus, Molluscum contagiosum virus, Monkeypox virus, Muerto Canyon virus, Mumps virus, Murray Valley encephalitis virus, Norwalk virus (and related viruses), O'nyong-nyong virus, Omsk hemorrhagic fever virus, Orf virus (contagious pustular dermatitis virus), Oropouche virus, Papillomaviruses 1-60, Parainfluenza viruses 1 and 3, Parainfluenza viruses 2 and 4, Parvovirus B-19, Polioviruses 1-3, Pseudocowpox virus (milker's nodule virus), RA-1 virus, Rabies virus, Respiratory syncytial virus, Rhinoviruses 1-113, and Rift Valley fever virus.

Staphylococcal abscesses; Staphylococcal pneumonia; Staphylococcal bacteremia; Staphylococcal osteomyelitis; Staphylococcal; Influenza viruses A, B, and C; Parainfluenza viruses 1-4; Mumps virus; Adenoviruses; Reoviruses; Respiratory syncytial virus; Epstein-Barr virus; Rhinoviruses; Polioviruses; Colorado tick fever; Phlebotomus fever; Venezuelen equine encephalitis; Rift valley fever; Dengue fever; West Nile fever; Barmah Forest virus; Chikungunya disease; Mayaro virus disease; Ross river virus disease; Sindbis virus disease (Okelbo disease, Pogosta disease, Karelian fever); Eastern equine encephalitis; Western equine encephalitis; St. Louis encephalitis; Venezuelen equine encephalitis; California virus group; Japanese encephalitis; Powassan virus; Murray Valley encephalitis; Kyasanur Forest disease; Tick-borne encephalitis virus; Lymphocytic choriomeningitis; Yellow fever; Dengue hemorrhagic fever; Kyasanur Forest disease; Omsk hemorrhagic fever; Crimean-Congo hemorrhagic fever; Hantaan virus; Seoul virus; Puumala virus; Machupo virus; Junin virus; Lassa fever; Marburg virus; Ebola virus; lasmodium spp; Trypanosoma spp; Microfilarie; Leishmania spp; naegleria Hartmannella Acanthamoeba group; Giardia lamblia, Strongyloides, Entamoeba histolytica, Schistosoma mansoni, Schistosoma japonicum; Entamoeba histolytica, Other amebas; Giardia lamblia; Cryptosporidium; Trichuris trichiura, Ascaris lumbricoides, Hookworm, Strongyloides, Tapeworm, Fluke; Enterobius vermicularis; Entamoeba

histolytica; Paragonimus westermani; Entamoeba histolytica, Strongyloides, Echinococcus granulosus, Hookworm, Ascaris spp, Pneumocystis carinii; Pneumocystis carinii; Onchocerca volvulus; Leishmania spp, Entamoeba histolytica; Taenia solium; Trichomonas spp; Schistosoma haematobium

Pathogens include but are not limited to: feline pathogen, canine pathogen, equine pathogen, bovine pathogen, avian pathogen, porcine pathogen, or human pathogen. Human pathogen includes but is not limited to: herpes simplex virus-1, herpes simplex virus-2, human cytomegalovirus, Epstein-Barr virus, Varicell-Zoster virus, human herpesvirus-6, human herpesvirus-7, human influenza, human immunodeficiency virus, rabies virus, measles virus, hepatitis B virus and hepatitis C virus. Furthermore, the antigenic polypeptide of a human pathogen may be associated with malaria or malignant tumor from the group consisting of Plasmodium falciparum, Bordetella.

Equine pathogen can derived from equine influenza virus or equine herpesvirus. Examples of such antigenic polypeptide are equine influenza virus type A/Alaska 91 neuraminidase, equine influenza virus type A/Prague 56 neuraminidase, equine influenza virus type A/Miami 63 neuraminidase, equine influenza virus type A/Kentucky 81 neuraminidaseequine herpesvirus type 1 glycoprotein B, and equine herpesvirus type 1 glycoprotein D.

Bovine pathogens include but are not limited to: bovine respiratory syncytial virus or bovine parainfluenza virus. The antigenic polypeptide of derived from bovine respiratory syncytial virus equine pathogen can derived from equine influenza virus is bovine respiratory syncytial virus attachment protein (BRSV G), bovine respiratory syncytial virus fusion protein (BRSV F), bovine respiratory syncytial virus nucleocapsid protein (BRSV N), bovine parainfluenza virus type 3 fusion protein, and the bovine parainfluenza virus type 3 hemagglutinin neuraminidase.

This invention provides a method for the detection of virus , comprising: a) obtaining a whole blood sample from the subject; b) incubating the whole blood sample in a culture in the presence of a media containing a mitogen with or without antigen, so as to induce polyclonal and specific activation of lymphocytic cells leading to antibody production; c) exposing the cells so as to separately recover nucleic acid sequences of the virus specific antibody; d) performing reverse transcription of an RNA of a virus specific antibodies in a sample to produce a DNA copy; e) performing polymerase chain reaction amplification of the DNA copy of the RNA genome to produce a plurality of DNA copies; f) performing hybridization of the DNA copies to a plurality of complementary DNA probes to detect the DNA content of the sample; thereby detecting virus infection.

This invention provides a kit for the detection of specific virus antigen from a subject, comprising: a container for collecting whole blood samples, wherein the container contains a media containing mitogen with or without antigen, effective to induce polyclonal and specific activation of lymphocytic cells leading to antibody production and an assay for the detection of the specific virus antibody.

The present invention also includes a kit comprising a blood collection container containing an effective concentration of mitogen with or without antigen therein. The container can optionally contain a culture medium. The preferred container is a test tube. The blood collection container can be plastic, glass, or any other material that is compatible with culturing blood. It is to be understood that the present invention also includes blood containing means other then a blood collection tube including, but not limited to, microtiter plates containing wells in which the blood can be incubated, tissue culture flasks, glass flasks such as an Erlenmeyer flask, and any other container in which the blood can be cultured.

This invention provides a method of diagnosing virus in a subject which comprises: a) obtaining a whole blood sample from the subject; b) incubating the

whole blood sample in a culture in the presence of a media containing a mitogen with or without antigen, to induce polyclonal and specific activation of lymphocytic cells; c) obtaining a nucleic acid sequence from lymphocytes; d) contacting the nucleic acid sequence with a labeled nucleic acid sequence of at least 15 nucleotides capable of specifically hybridizing with the isolated DNA of the specific antibody, under hybridizing conditions; and e) determining the presence of the nucleic acid sequence hybridized, the presence of which is indicative of virus in the subject, thereby diagnosing virus in the subject.

This invention provides an *in vitro* method for use as a diagnostic/prognostic indicator of a subject infected/exposed to a virus, comprising the following steps: a) obtaining a whole blood sample from the subject; b) incubating the whole blood sample in a culture in the presence of a mitogen with or without antigen containing media,so as to induce polyclonal and specific activation of lymphocytic cells leading to antibody production; c) exposing the resultant culture of step b) to an virus antigen, thereby allowing an antigen-antibody immune complex to form; d) detecting the amount of antigen-antibody immune complex of step c); comparing the amount of the complex to a second amount of complex previously detected, the increase of such amount is indicative of a subject seroconvereting. In one embodiment, the amounts of antibodies, either quantitatively, i.e. level of antibody titre, or qualitatively, from one time point to another are compared.

In one embodiment, the DNA sequence from the virus is amplified before step (b). In another embodiment PCR is employed to amplify the nucleic acid sequence. Methods of amplifying nucleic acid sequences are known to those skilled in the art.

Primer pairs for HIV are known to those skilled in the art . For example, the following may be used:

SK 38/39 - (probe SK19) gag

SK	63/69	
SK	101/145	pol
SK68-/ Benvb		env
B163 env/ Benvend		env

In one embodiment, the viral enzyme is reverse transcriptase (RT). In another embodiment the viral enzyme is protease. In another embodiment the enzyme is integrase. Other enzymes are known to those skilled in the art.

Examples of such antigens and specific antibodies which are immunoreactive with sera from patients infected with parenterally transmitted non-A, non-B hepatitis (PT-NANBH) virus, to polynucleotide sequences which encode the peptides, to an expression system capable of producing the peptides, and to methods of using the peptides for detecting PT-NANBH infection in human sera are known to those skilled in the art. Specifically, this invention contemplates use of the HCV genome core, NS4 regions, surface-bound HCV antigen, 409-1-1 antigens (409-1-1(abc), 409-1-1(c-a), and related antigens. Yoshikura, Hiroshi, et al, U.S. Patent No. 5,552,310 Replication of Hepatitis C virus genome and identification of virus having high infectivity; Reyes, Gregory,et al., U.S. Patent No. 5,538,865, 5,443,965 and 5,436,318 Hepatitis C virus epitopes; Resnick, Robert M., et al., U.S.Patent No. 5,527,669, Methods, primers and probes for detection of hepatitis C and novel variants; and Wang, Chang Y., U.S. Patent No. 5,436,126 Synthetic peptides specific for the detection of specific antibodies to HCV, diagnosis of HCV infection.

Further, antigens are known to those skilled in the art. For example, the following antigens may be used SOD/HCV polypeptide c100-3 (363 AA); Polypeptide 2nd; and capsoid peptides.

As defined herein "sample" refers to any sample obtained from an organism. Examples of biological samples include body fluids and tissue specimens. The source of the sample may be derived from such physiological media as blood,

serum, plasma, breast milk, pus, tissue scrapings, washings, urine, tissue, such as lymph nodes, spleen or the like.

The subjects may be a mammal, or more specifically a human, horse, pig, rabbit, dog, sheep, goat, monkey, cow, cat, or rodent. In the preferred embodiment the subject is a human.

"Mitogens" as defined herein means any material that activates lymphocytic cells, so as to secrete or produce specific antibodies. In one embodiment the mitogen is a B cell activator, such as pokeweed mitogen, a lectin, a bacterial endotoxin, an antigen, lipid A, or a lymphokine. In another embodiment the mitogen is a superantigen such as a toxin from bacteria which include staphylococci and staphylococci A toxins (30KD toxins), enterotoxins A,B,C1,C2,D,E (from Staphylococcus aureus), exotoxins A,B,C, and exfoliative toxin A,B. In another embodiment, the mitogen is a gram-negative LPS sequence. In another embodiment the mitogen is a peptidoglycan from both gram negative and gram positive bacteria, for example, toxic shock syndrome toxin TSST-1, ExFT, MAM, Strep M., or a Gram-negative lipopolysacchride (LPS) sequences. In another embodiment the mitogen is herpesviruses such as Epstein-Barr Virus(EBV), a retrovirus, mouse mammary tumor virus (MMTV), picornavirus(rats) Coxsackie virus, mumps and measles viruses and Mtv virus (1-9,11,13,43). In another embodiment the mitogen is heat shock proteins(HSP). In another embodiment the mitogen is an antibody which includes but is not limited to: Anti CD3 antibodies, Anti TCR (T cell Receptor), Anti IgM, Anti IgD, Anti CD28 in both soluble form or bound. It is contemplated that interleukines or cytokines, such as IL-4, either alone or in conjunction with additional factors may be added. In another embodiment the mitogen is phorbol ester such as phorbol myristate acetate, PMA with calcium ionophore and IL-4. In another embodiment pharmacological activators (such as diacylglycerol) that work through paths such as the PIP_2 derived second messenger path. In another embodiment the mitogen is a lectin including Pokeweed mitogen (PWM) and similar acting mitogens with

or without antigens. In the preferred embodiment Pokeweed mitogen is used. Pokeweed mitogen includes pure and crude extracts from Phylotacca Americana.

As used herein, "whole blood" means blood collected from an animal or human. The whole blood contains red blood cells which may be lysed while maintaining the viability of the remaining white blood cells. Whole blood may be collected with heparin, EDTA, citrate or any other substance that prevents coagulation and clotting.

In one embodiment, the optimal concentration of mitogen with or without antigen is easily determined without undue experimentation by one of ordinary skill in the art. With regard to the preferred mitogen, pokeweed mitogen, the preferred concentration range is between approximately 1:100 and 1:1600 dilutions of stock PWM. The most preferred concentration range is between approximately 1:200 and 1:1:400 dilutions of stock PWM. The preferred source of the stock PWM is GIBCO, New York, New York. The lyophilized PWM is reconstituted with 5 ml of distilled water to make the stock solution.

The concentration of pokeweed mitogen may range from about 0.025-50.0 ul/ml. The concentration range may be from about 0.1-0.5 ul/ml. The preferred concentration is 0.2 ul/ml. If the mitogen is Wheat germ agglutination the concentration range is about 0.1-2.5 ul/ml. If the mitogen is Sac Cowan I mitogen the concentration range is 1:200-1:2000 dilution.

As defined herein, "culture medium" means any medium than can be used to sustain a sample to practice the present invention, including but not limited to RPMI 1640 (GIBCO, New York, New York) with or without fetal calf serum, preferably supplemented with appropriate antibiotics and glutamine. Other culture media which may be used in practicing the present invention include, but are not limited to, Eagles, Dulbecco's, McCoy's, Media 199, Waymouth's media, and serum free medium with or without supplement. In another embodiment the mitogen is without media.

The nucleic acid sequence referred herein may be DNA, RNA or cDNA. Amplification is carried out using the polymerase chain reaction and a single or plurality of primer sets so as to provide PCR products of different lengths. The plurality of primer sets are amplified together by PCR. Each primer set is amplified separately by PCR.

If one employs two different primer sets, the present invention contemplates that the first primer set is capable of generating a product of a length short enough to be essentially transparent to the addition of the addition compounds to the nucleic acid under a defined set of amplification conditions. That is regardless of the efficiency of the covalent addition of addition compounds, the length of the product is sufficient such that amplification product will be detected.

The second primer set is also capable of generating a product of a length long enough to be affected-not completely inhibited but inhibited in part-by the addition of the addition compounds to the nucleic acid of the nucleic acid-containing antibodies with the same amplification conditions as above. This being so the efficiency of the covalent addition of addition compounds will be reflected in the amount of amplification product detected.

Where three different primer sets are used, the present invention contemplates that the first primer set is capable of generating a product of a length short enough to be essentially transparent to the addition of the addition compounds to the nucleic acid under a defined set of amplification conditions. In other words regardless of the efficiency of the covalent addition of addition compounds, the length of the product is sufficient such that amplification product will be detected. The second primer set is also capable of generating a product of a length long enough to be affected-not completely inhibited but inhibited in part-by the addition of the addition compounds to the nucleic acid of the nucleic acid-containing antibodies with the same amplification conditions as above. Again the efficiency of the covalent addition of addition compounds will be reflected in the amount of amplification product detected. The third primer set

is capable of generating a product of a length long enough to be completely inhibited by the addition of addition compounds to the nucleic acid of the nucleic-acid containing antibodies. Covalent addition of the addition compounds will be reflected by the complete absence of measurable amplification product.

For example, after amplification, a portion of the PCR reaction mixture can be separated and subjected to hybridization with an end-labeled nucleotide probe, such as a 32P labeled adenosine triphosphate end-labeled probe. In PCR, an end-labeled oligonucleotide probe hybridizes in solution to a region of the amplified sequence and, in the process, reconstitutes a specific endonuclease site. Thus, hybridization of the labeled probe with the amplified sequence yields a double-stranded DNA form that is sensitive to selective restriction enzyme digestion. After restriction with an endonuclease, the resulting samples can be analyzed on a polyacrylamide gel, and autoradiograms of the portion of the gel with the diagnostic labeled fragment can be obtained. The appearance of a diagnostic fragment (e.g. 10-15 bases in length) in the autoradiogram indicates the presence of tumor antigen associated antibodies nucleotide sequences in the PBMCs.

"PCR" refers to a process of amplifying one or more specific nucleic acid sequences, wherein (1) oligonucleotide primers which determine the ends of the sequences to be amplified are annealed to single-stranded nucleic acid in a test sample, (2) a nucleic acid polymerase extends the 3' ends of the annealed primers to create a nucleic acid strand complementary in sequence to the nucleic acid to which the primers were annealed, (3) the resulting double-stranded nucleic acid is denatured to yield two single-stranded nucleic acids, and (4) the processes of primer annealing, primer extension, and product denaturation are repeated enough times to generate easily identified and measured amounts of the sequences defined by the primers. Practical control of the sequential annealing, extension, and denaturation steps is exerted by varying the temperature of the reaction container, normally in a repeating cyclical manner. Annealing and extension occur optimally in the 40o C. to 80o C. temperature range (exact value

depending on primer concentrations and sequences), whereas denaturation requires temperatures in the 80o C. to 100o C. range (exact value depending on target sequence and concentration).

DNA amplification procedures by PCR are well known and are described in U.S. Pat. Nos. 4,683,195, 4,683,202, and 4,965,188, each of which is incorporated herein by reference. For ease of understanding the advantages provided by the present invention, a summary of PCR is provided. PCR requires two primers that hybridize with the double-stranded target nucleic acid sequence to be amplified. In PCR, this double-stranded target sequence is denatured and one primer is annealed to each strand of the denatured target. The primers anneal to the target nucleic acid at sites removed from one another and in orientations such that the extension product of one primer, when separated from its complement, can hybridize to the other primer. Once a given primer hybridizes to the target sequence, the primer is extended by the action of a DNA polymerase. The extension product is then denatured from the target sequence, and the process is repeated. One particular method for minimizing the effects of cross contamination of nucleic acid amplification is described in U.S. Pat. No. 5,035,996, which is incorporated herein by reference.

The PCR technique useful for determining whether seropositive or seronegative persons have detectable levels of specific antibodies. In PCR techniques, oligonucleotide primers to the specific tumor associated antibody is indicative of the subject having cancer.

Tumor antigen associated antibodies produced complementary to the two 3' borders of the DNA region to be amplified are synthesized. The polymerase chain reaction is then carried out using the two primers. See *PCR Protocols: A Guide to Methods and Applications* [PCR Protocols: A Guide to Methods and Applications. (1990) Innis, M., Gelfand D., Sninsky, J. and White, T., eds., Academic Press, San Diego]. Following PCR amplification, the PCR-amplified regions of an tumor antigen associated antibodies can be tested for their ability

to hybridize to the three specific nucleic acid probes listed above. Alternatively, hybridization of nucleic acid of the tumor antigen associated antibodies to the above nucleic acid probes can be performed by a Southern blot procedure without DNA amplification and under stringent hybridization conditions as described herein. U.S. Patent No. 5,494,810 Barany, Francis,et al. "Polymerase chain reaction (PCR)" refers to a patented process (described in U.S. Patent Nos. 4,683,202 and 4,683,195) for the exponential amplification of aspecific DNA fragment by utilizing two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in a target DNA are incorporated by reference. Also, those assays disclosed in the disclosures of the following U.S. patents: U.S. Pat. Nos. 4,459,359 is incorporated by reference.

Choosing PCR primer sequences, preparing PCR reagents and reaction mixtures, and designing and running PCR reactions are well known procedures in the PCR art. In the event that nucleic acid amplification is performed on suspended cells in a standard PCR tube, the cells are treated like any conventional PCR test sample: diluted into reaction mixture shortly before amplification is started, at a total cell number ranging from approximately 100 to approximately 10^6 .

If multiple samples are amplified simultaneously in different tubes, a fresh sampler tip is used to add the missing reagent(s) to each tube, to prevent cross-contamination. After all tubes have been prepared and capped, the standard three-temperature thermal cycle program of denaturation, annealing, and extension for approximately 10 to 40 cycles is performed under thermal cycler microprocessor control. Alternatively, and often preferably, a series of two-temperature cycles can be run wherein annealing and extension are performed at a single temperature, normally optimized for stringent annealing of primer to template. Because reaction rates may be somewhat retarded with cellular preparations as compared to cell-free nucleic acids, it may be necessary to increase the durations of the denaturation, anneal, extend, or anneal-extend cycle segments as much as several-fold from values standard when the test sample contains cell-free nucleic acid. This adjustment is easily performed by

looking for conditions which maximize the intensity of the signal seen during amplified nucleic acid detection or which minimize the number of cycles needed to reach a given signal intensity. A similar optimization procedure can be used for MgCl2, dNTP, primer, and enzyme concentrations in the reaction mixture; these parameters often show different optima for different targets, and also may be affected when amplification occurs within fixed cells.

Primer pairs of known sequence positioned 10-300 base pairs apart that are complementary to the plus and minus strands of the DNA to be amplified can be prepared by well known techniques for the synthesis of oligonucleotides. One end of each primer can be extended and modified to create restriction endonuclease sites when the primer is annealed to the nucleic acid sequence of specific antibodies. The PCR reaction mixture can contain the DNA of the specific antibodies, the DNA primer pairs, four deoxyribonucleoside triphosphates, MgCl2, DNA polymerase, and conventional buffers The DNA can be amplified for a number of cycles. It is generally possible to increase the sensitivity of detection by using a multiplicity of cycles, each cycle consisting of a short period of denaturation of the DNA of the specific antibodies at an elevated temperature, cooling of the reaction mixture, and polymerization with the DNA polymerase. Oligonucleotide primers and probes are known to those skilled in the art.

Oligonucleotides for use as probes or PCR primers are chemically synthesized according to the solid phase phosphoramidite triester method first described by Beaucage and Carruthers [Beaucage and Carruthers (1981) Tetrahedron Lett. 22:1859-1862.] using an automated synthesizer, as described in Needham-VanDevanter [Needham-VanDevanter, D.R., et al., (1984) Nucleic Acids Res. 12:6159-6168]. Purification of oligonucleotides is by either native acrylamide gel electrophoresis or by anion-exchange HPLC as described in Pearson, J.D. and Regnier, F.E. [Pearson, J.D., and Regnier, F.E., (1983) J. Chrom. 255:137-14976.]. The sequence of the synthetic oligonucleotide can be verified using the chemical degradation method of Maxam, A.M. and Gilbert, W.

[Maxam, A.M. and Gilbert, W. Methods in Enzymology (1980) Grossman, L. and Moldave, D., eds., Academic Press, New York, 65:499-560.]. Tavernarakis, N., U.S. Patent No. 5,569,582 is directed to amplification and detection of tumor antigen associated antibodies nucleic acid is hereby incoporated by reference.

The present invention has applicability to diagnosing a subject having cancer by the amplification and subsequent detection of nucleic acid sequences of anti tumor antigen associated antibodies. "Amplification" is a special case of nucleic acid replication involving template specificity. It is to be contrasted with non-specific template replication (i.e., replication that is template-dependent but not dependent on a specific template). Template specificity is here distinguished from fidelity of replication (i.e., synthesis of the proper polynucleotide sequence) and nucleotide (ribo- or deoxyribo-) specificity. Template specificity is frequently described in terms of "target" specificity. Target sequences are "targets" in the sense that they are sought to be amplified or detected preferentially in the presence of other non-target nucleic acid sequences. Amplification techniques have been designed primarily for the detection of specific target sequences. Template specificity is achieved, in most amplification techniques, by the choice of enzyme. Amplification enzymes are enzymes that, under the conditions in which they are used, will process only specific sequences of nucleic acid in a heterogenous mixture of nucleic acid.

In the case of T4 DNA ligase, the enzyme will not ligate the two oligonucleotides where there is a mismatch between the oligonucleotide substrate and the template at the ligation junction. D. Y. Wu and R. B. Wallace, Genomics 4:560 (1989). Finally, Taq polymerase, by virtue of its ability to function at high temperature, is found to display high specificity for the sequences bounded and thus defined by the primers; the high temperature results in thermodynamic conditions that favor primer hybridization with the specific target sequences and not hybridization with non-target sequences. R. K. Saiki in PCR Technology,

Principles and Applications for DNA Amplification (H. A. Erlich, Ed.), pp. 7-16 (1989).

Some amplification techniques take the approach of amplifying and then detecting target; others detect target and then amplify probe. Regardless of the approach, the sample containing nucleic acid must be free of inhibitors for amplification to occur at high efficiency.

Amplification "reagents" are defined as those reagents (primers, deoxyribonucleotide triphosphates, etc.) needed for amplification except for nucleic acid and the amplification enzyme. Typically, amplification reagents along with other reaction components are placed and contained in a reaction vessel (test tube, microwell, etc.).

The preferred lysing agent is protease K. Protease K is a proteolytic enzyme from Tritirachium album. It is particularly useful in the present invention because it has no significant DNase activity and, therefore, does not degrade nucleic acid which would prevent amplification. It is also attractive because it is inexpensive and commercially available (e.g., Sigma, St. Louis, Mo.,U.S.A., catalogue No. p4914 "Proteinase K"). Various treatment conditions using protease K have been found useful. It is preferred that a high concentration of protease K (e.g., 1.5-2.5 mg/ml) be used for short (5-10 minutes) incubation periods to completely degrade cellular and tumor antigen associated antibodies. When lower concentrations of protease K (e.g., 0.5 mg/ml) are used, longer incubation periods (30-60 minutes) are required to achieve the same effect. Other lysis approaches are also contemplated, including lysis by heating.

Detection" of PCR-amplified nucleic acid refers to the process of observing, locating, or quantitating an analytical signal which is inferred to be specifically associated with the product of PCR amplification, as distinguished from PCR reactants. The analytical signal can result from visible or ultraviolet absorbance or fluorescence, chemiluminescence, or the photographic or autoradiographic

image of absorbance, fluorescence, chemiluminescence, or ionizing radiation. Detection of in situ PCR products involves microscopic observation or recording of such signals. The signal derives directly or indirectly from a molecular "tag" attached to a PCR primer or dNTP or to a nucleic acid probe, which tag may be a radioactive atom, a chromophore, a fluorophore, a chemiluminescent reagent, an enzyme capable of generating a colored, fluorescent, or chemiluminescent product, or a binding moiety capable of reaction with another sequence or particle which directly carries or catalytically generates the analytical signal. Common binding moieties are biotin, which binds tightly to streptavidin or avidin, digoxigenin, which binds tightly to anti-digoxigenin antibodies, and fluorescein, which binds tightly to anti-fluorescein antibodies. The avidin, streptavidin, and antibodies are easily attached to chromophores, fluorophores, radioactive atoms, and enzymes capable of generating colored, fluorescent, or chemiluminescent signals.

RNA is prepared by any number of methods; the choice may depend on the source of the sample and availability. Methods for preparing RNA are described in Davis et al., 1986, Basic Methods in Molecular Biology, Elsevier, NY, Chapter 11; Ausubel et al., 1987, Current Protocols in Molecular Biology, Chapter 4, John Wiley and Sons, NY; Kawasaki and Wang, 1989, PCR Technology, ed. Erlich, Stockton Press NY; Kawasaki, 1990, PCR Protocols: A Guide to Methods and Applications, Innis et al. eds. Academic Press, San Diego; and Wang and Mark, 1990, PCR Protocols: A Guide to Methods and Applications, Innis et al. eds. Academic Press, San Diego; all of which are incorporated herein by reference. As noted above, the probe will be capable of specific hybridization to a specific tumor antigen associated antibody nucleic acid is indicative of the subject having cancer.

Such "specific hybridization" occurs when a probe hybridizes to a target nucleic acid, as evidenced by a detectable signal, under conditions in which the probe does not hybridize to other nucleic acids (*e.g.*, animal cell or other bacterial nucleic acids) present in the sample. A variety of factors including the length and

base composition of the probe, the extent of base mismatching between the probe and the target nucleic acid, the presence of salt and organic solvents, probe concentration, and the temperature affect hybridization, and optimal hybridization conditions must often be determined empirically. For discussions of nucleic acid probe design and annealing conditions, see, for example, Ausubel, F., *et al.*, Methods in Enzymology [Methods in Enzymology Vol. 152, (1987) Berger, S. and Kimmel, A. ed., Academic Press, New York] or *Hybridization with Nucleic Acid Probes* all of which are incorporated herein by reference.

High stringent hybridization conditions are selected at about 5° C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength and pH. The Tm is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typically, stringent conditions will be those in which the salt concentration is at least about 0.02 molar at pH 7 and the temperature is at least about 60°C. As other factors may significantly affect the stringency of hybridization, including, among others, base composition and size of the complementary strands, the presence of organic solvents, ie. salt or formamide concentration, and the extent of base mismatching, the combination of parameters is more important than the absolute measure of any one. For Example high stringency may be attained for example by overnight hybridization at about 68°C in a 6x SSC solution, washing at room temperature with 6x SSC solution, followed by washing at about 68°C in a 6x SSC in a 0.6x SSX solution.

Hybridization with moderate stringency may be attained for example by: 1) filter pre-hybridizing and hybridizing with a solution of 3x sodium chloride, sodium citrate (SSC), 50% formamide, 0.1M Tris buffer at Ph 7.5, 5x Denhardt's solution; 2.) pre-hybridization at 37°C for 4 hours; 3) hybridization at 37°C with amount of labeled probe equal to 3,000,000 cpm total for 16 hours; 4) wash in 2x SSC and 0.1% SDS solution; 5) wash 4x for 1 minute each at room temperature at 4x at 60°C for 30 minutes each; and 6) dry and expose to film.

The phrase "selectively hybridizing to" refers to a nucleic acid probe that hybridizes, duplexes or binds only to a particular target DNA or RNA sequence when the target sequences are present in a preparation of total cellular DNA or RNA. By selectivly hybridizing it is meant that a probe binds to a given target in a manner that is detectable in a different manner from non-target sequence under high stringency conditions of hybridization. in a different "Complementary" or "target" nucleic acid sequences refer to those nucleic acid sequences which selectivly hybridize to a nucleic acid probe. Proper annealing conditions depend, for example, upon a probe's length, base composition, and the number of mismatches and their position on the probe, and must often be determined empirically. For discussions of nucleic acid probe design and annealing conditions, see, for example, Sambrook *et al.*, [Sambrook, et al. (1989) Molecular Cloning: A Laboratory Manual (2nd ed.), Cold Spring Harbor Laboratory, Vols. 1-3.] or Ausubel, F., *et al.*, [Ausubel, F., et al. (1987) Current Protocols in Molecular Biology, New York.].

DNA or mRNA of tumor antigen associated antibodies may be detected by Southern blotting, single stranded conformational polymorphism gel electrophoresis (SSCP), PCR or other DNA based techniques, or for RNA species by Northern blotting, PCR or other RNA-based techniques.

As defined herein nucleic acid probes may be DNA or RNA fragments. DNA fragments can be prepared, for example, by digesting plasmid DNA, or by use of PCR, or synthesized by either the phosphoramidite method described by Beaucage and Carruthers, or by the triester method according to Matteucci, *et al.*, [Matteucci, et al. (1981) Am. Chem. Soc. 103:3185.], both incorporated herein by reference. A double stranded fragment may then be obtained, if desired, by annealing the chemically synthesized single strands together under appropriate conditions or by synthesizing the complementary strand using DNA polymerase with an appropriate primer sequence. Where a specific sequence for a nucleic acid probe is given, it is understood that the complementary strand is also identified and included. The complementary strand will work equally well

in situations where the target is a double-stranded nucleic acid. It is also understood that when a specific sequence is identified for use a nucleic probe, a subsequence of the listed sequence which is 25 base pairs or more in length is also encompassed for use as a probe.

The antibody or DNA sequence may be labeled with a detectable marker including, but not limited to: a radioactive label, or a colorimetric, a luminescent, or a fluorescent marker, or gold. Radioactive labels include, but are not limited to: ^{3}H, ^{14}C, ^{32}P, ^{33}P, ^{35}S, ^{36}Cl, ^{51}Cr, ^{57}Co, ^{59}Co, ^{59}Fe, ^{90}Y, ^{125}I, ^{191}I, ^{186}Re, and ^{188}Re. Fluorescent markers include but are not limited to: fluorescein, rhodamine and auramine. Colorimetric markers include, but are not limited to: biotin, and digoxigenin. Methods of producing the polyclonal or monoclonal antibody are known to those of ordinary skill in the art.

Further, the antibody or nucleic acid sequence complex may be detected by a second antibody which may be linked to an enzyme, such as alkaline phosphatase or horseradish peroxidase. Other enzymes which may be employed are well known to one of ordinary skill in the art.

The tumor antigens or antibodies may also be labeled using fluorescent labels, enzyme labels, free radical labels, or bacteriophage labels, using techniques known in the art. Typical fluorescent labels include fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, alophycocyanin, and Texas Red.

Since specific enzymes may be coupled to other sequences by covalent links, the possibility also exists that they might be used as labels for the production of tracer materials. Suitable enzymes include alkaline phosphatase, beta-galactosidase, glucose-6-phosphate dehydrogenase, maleate dehydrogenase, and peroxidase. Types of enzyme immunoassay are the enzyme-linked immunosorbent assay (ELISA), ELISPOT, and the homogeneous enzyme immunoassay, also known as enzyme-multiplied immunoassay (EMIT, Syva Corporation, Palo Alto, CA). In the ELISA system, separation may be achieved,

for example, by the use of antibodies coupled to a solid phase. The EMIT system depends on deactivation of the enzyme in the tracer-antibody complex; the activity can thus be measured without the need for a separation step.

Additionally, chemiluminescent compounds may be used as labels. Typical chemiluminescent compounds include luminol, isoluminol, aromatic acridinium esters, imidazoles, acridinium salts, and oxalate esters. Similarly, bioluminescent compounds may be utilized for labeling, the bioluminescent compounds including luciferin, luciferase, and aequorin.

Once labeled, the antibody may be employed to identify and quantify immunologic counterparts (antibody or tumor antigenic polypeptide) utilizing techniques well-known to the art. Antibodies include but are not limited to: IgG and subsets, IgA, IgE, IgM, IgD.

The phrase "specifically binds to an tumor antigen associated antibodies" or "specifically immunoreactive with", when referring to the tumor antigen associated antibodies, refers to a binding reaction which is determinative of the presence of the tumor antigen associated antibody. Thus, under designated immunoassay conditions, the specified tumor antigen associated antibody binds to the tumor antigen associated antibody and does not bind in a significant amount to other antibodies present in the sample. Specific binding to an tumor antigen associated antibody under such conditions may require an tumor antigen that is selected for its specificity for a particular protein.

A variety of immunoassay formats may be used to select specific antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immunoreactive with a protein. See Harlow and Lane [Harlow and Lane, (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Publication, New York.] for a description of immuno assay formats and conditions that can be used to determine specific immunoreactivity.

A description of a radioimmunoassay (RIA) may be found in *Laboratory Techniques in Biochemistry and Molecular Biology* [Laboratory Techniques in Biochemistry and Molecular Biology (1978) North Holland Publishing Company, New York.], with particular reference to the chapter entitled "An Introduction to Radioimmune Assay and Related Techniques" by Chard, T., incorporated by reference herein.

A description of general immunometric assays of various types can be found in the following U.S. Pat. Nos. 4,376,110 (David *et al.*) or 4,098,876 (Piasio) and 4,870,003 (Kortright) are incorporated by reference.

The presence or concentration of this antigen-antibody complex is determined to detect or quantitate the presence of tumor antigen associated antibodies in the biological sample.

The ligand to the peptide sequence may carry a specific substance such as a metal ion (iron or zinc or other) into the tumor and thus serve as a means to deliver toxic substances (radioactive or cytotoxic chemical i.e. toxin like ricin or cytotoxic alkylating agent or cytotoxic prodrug) to the tumor. The linkage of the antibody and the toxin or radioisotope can be chemical. Examples of direct linked toxins are doxorubicin, chlorambucil, ricin, pseudomonas exotoxin etc., or a hybrid toxin can be generated ½ with specificity for peptide and the other ½ with specificity for the toxin. Such a bivalent molecule can serve to bind to the tumor and the other ½ to deliver a cytotoxic to the tumor or to bind to and activate a cytotoxic lymphocyte such as binding to the T_1 - T_3 receptor complex. Antibodies of required specificity can also be cloned into T cells and by replacing the immunoglobulin domain of the T cell receptor (TcR); cloning in the desired MAb heavy and light chains; splicing the U_h and U_L gene segments with the constant regions of the α and ß TCR chains and transfecting these chimeric Ab/TcR genes in the patients' T cells, propagating these hybrid cells and infusing them into the patient. Specific knowledge of tissue specific antigens for targets

and generation of MAb's specific for such targets will help make this a usable approach.

It is possible to produce a toxic genetic chimera such as TP-40 a genetic recombinant that possesses the cDNA from TGF-alpha and the toxic portion of pseudomonas exotoxin so the TGF and portion of the hybrid binds the epidermal growth factor receptor (EGFR) and the pseudomonas portion gets taken up into the cell enzymatically and inactivates the ribosomes ability to perform protein synthesis resulting in cell death.

Detecting the reaction of the antibody (or ligand) with the tumor antigen can be facilitated by the use of an antibody or ligand that is labeled with a detectable moiety by methods known in the art. Such a detectable moiety will allow visual detection of a precipitate or a color change, visual detection by microscopy, or automated detection by spectrometry or radiometric measurement or the like. Examples of detectable moieties include fluorescein and rhodamine (for fluorescence microscopy), horseradish peroxidase (for either light microscopy or electron microscopy and biochemical detection), biotin-strepavidin (for light or electron microscopy) and alkaline phosphatase (for biochemical detection by color change). The detection methods and moieties used can be selected, for example, from the list above or other suitable examples by the standard criteria applied to such selections (Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1988).

Enzyme immunoassays such as immunofluorescence assays (IFA), photometric assays, enzyme linked immunosorbent assays (ELISA), ELISPOT, and immunoblotting can be readily adapted to accomplish the detection of the specific antibodies. An ELISA method effective for the detection of the tumor antigen associated antibodies can, for example, be as follows: (1) bind the tumor antigen to a substrate; (2) contact the bound tumor antigen with a biological sample, such as a bodily fluid before and after culture or tissue sample or lymphocytes before and after culture containing the antibody; (3) contact the

above with a secondary antibody bound to a detectable moiety (e.g., horseradish peroxidase enzyme or alkaline phosphatase enzyme); (4) contact the above with the substrate for the enzyme; (5) contact the above with a color reagent; (6) observe the color change.

Where the tumor antigens are labeled, the labels can include radioisotopes, fluorophores, enzymes, luminescers or particles. These and other labels are well known in the art and are described, for example, in the following U.S. Pat. Nos. 3,766,162; 3,791,932; 3,817,837; 3,996,345; and 4,233,402. Assays employing the tumor antigen associated antibodies from the cell lines can be heterogenous, i.e., requiring a separation step, or homogenous. If the assay is heterogenous a variety of separation means can be employed, including centrifugation, filtration, chromatography or magnetism.

Other detection systems which may also be used include those based on the use of protein A derived from Staphylococcus aureus Cowan strain I, protein G from group C Streptococcus sp. (strain 26RP66), or systems which employ the use of the biotin-avidin binding reaction.

Another method of immunoenzymatic detection of the presence of antibodies is the Western blot. The tumor antigens are separated electrophoretically and transferred to a nitrocellulose membrane or other suitable support. The body fluid to be tested is then brought into contact with the membrane and the presence of the immune complexes formed is detected by the method already described. In a variation on this methods, purified tumor antigen associated antibodies is applied in lines or spots on a membrane and allowed to bind. The membrane is subsequently brought into contact with the body fluid before and after culture to be tested and the immune complexes formed are detected using the previously described techniques.

The presence of specific antibodies in body fluid before and after culture may also be detected by agglutination. Tumor antigen associated antibodies lysates,

tumor antigen or purified composition referred to according to this invention, is used to coat, for example, latex particles which form an uniform suspension. When mixed with serum containing specific antigens to the antibodies present, the latex particles are caused to agglutinate and the presence of large aggregates can be detected visually.

Tumor antigen associated antibodies reactive with specific antibodies of tumor antigen can also be measured by a variety of immunoassay methods. For a review of immunological and immunoassay procedures applicable to the measurement of antibodies by immunoassay techniques, see *Basic and Clinical Immunology* 7th Edition [Basic and Clinical Immunology 7th Edition D. Stites and A. Terr ed.].

Hemagglutination Inhibition (HI) and Complement Fixation (CF) which are two laboratory tests that can be used to detect tumor antigen associated antibodies by testing for the presence of antibodies.

A person of ordinary skill in the art will be able to obtain appropriate DNA sample for diagnosing the subject. The DNA sample obtained by the above described method may be cleaved by restriction enzyme. The uses of restriction enzymes to cleave DNA and the conditions to perform such cleavage are well-known in the art.

In the above described methods, a size fractionation may be employed which is effected by a polyacrylamide gel. In one embodiment, the size fractionation is effected by an agarose gel. Further, transferring the DNA fragments into a solid matrix may be employed before a hybridization step. One example of such solid matrix is nitrocellulose paper.

Similarly, a Northern transfer may be used for the detection of message in samples of RNA or reverse transcriptase PCR and cDNA can be detected by methods described above. This procedure is also well known in the art.

An alternative means for determining the presence specific antibodies produced as a result of infection or exposure of the tumor antigen associated antibodies is *in situ* hybridization, or more recently, in situ polymerase chain reaction. In situ PCR is described in Neuvo *et al.* [Neuvo, et al. (1993) American Journal of Surgical Pathology 17(7), 683-690.], Intracellular localization of polymerase chain reaction (PCR)-amplified tumor antigen cDNA; Bagasra *et al.* [Bagasra, et al. (1992) J. New England Journal of Medicine 326(21):1385-1391.], Detection of tumor antigen by in situ polymerase chain reaction; and Heniford *et al.* [Heniford, et al. (1993) Nucleic Acids Research 21(14):3159-3166.], Variation in cellular EGF receptor mRNA expression demonstrated by in situ reverse transcriptase polymerase chain reaction. *In situ* hybridization assays are well known and are generally described in *Methods Enzymol.* incorporated by reference herein. In an *in situ* hybridization, cells are fixed to a solid support, typically a glass slide. The cells are then contacted with a hybridization solution at a moderate temperature to permit annealing of target-specific probes that are labeled. The probes are preferably labeled with radioisotopes or fluorescent reporters.

The above described probes are also useful for in-situ hybridization or in order to locate tissues which express this gene, or for other hybridization assays for the presence of this gene or its mRNA in various biological tissues. In-situ hybridization is a sensitive localization method which is not dependent on expression of tumor antigens or native vs. denatured conditions.

Oligonucleotides are 3'end-labeled with [α-^{35}S]dATP to specific activities in the range of 1 x 10^{10} dpm/ug using terminal deoxynucleotidyl transferase. Unincorporated labeled nucleotides are removed from the oligo probe by centrifugation through a Sephadex G-25 column or by elution from a Waters Sep Pak C-18 column.

A probe can be identified as capable of hybridizing specifically to its target nucleic acid by hybridizing the probe to a sample treated according the protocol

of this invention where the sample contains both target tumor antigen associated antibodies and animal cells (*e.g.*, nerve cells). A probe is specific if the probe's characteristic signal is associated with the tumor antigen associated antibodies DNA in the sample and not generally with the DNA of the host cells and non-biological materials (*e.g.*, substrate) in a sample.

The following stringent hybridization and washing conditions will be adequate to distinguish a specific probe (*e.g.*, a fluorescently labeled DNA probe) from a probe that is not specific: incubation of the probe with the sample for 12 hours at 37°C in a solution containing denatured probe, 50% formamide, 2X SSC, and 0.1% (w/v) dextran sulfate, followed by washing in 1X SSC at 70°C for 5 minutes; 2X SSC at 37°C for 5 minutes; 0.2X SSC at room temperature for 5 minutes, and H_2O at room temperature for 5 minutes. Those of skill will be aware that it will often be advantageous in nucleic acid hybridizations (*i.e.*, *in situ*, Southern, or other) to include detergents (*e.g.*, sodium dodecyl sulfate), chelating agents (*e.g.*, EDTA) or other reagents (*e.g.*, buffers, Denhardt's solution, dextran sulfate) in the hybridization or wash solutions.

It will be apparent to those of ordinary skill in the art that a convenient method for determining whether a probe is specific for a tumor antigen associated antibodies nucleic acid utilizes a Southern blot (or Dot blot) using DNA prepared from one or more tumor antigen associated antibodies. Briefly, to identify a target specific probe DNA is isolated from the tumor antigen associated antibodies. Test DNA is transferred to a solid (*e.g.*, charged nylon) matrix. The probes are labeled following conventional methods. Following denaturation and/or prehybridization steps known in the art, the probe is hybridized to the immobilized DNAs under stringent conditions. Stringent hybridization conditions will depend on the probe used and can be estimated from the calculated T_m (melting temperature) of the hybridized probe (see, *e.g.*, Sambrook for a description of calculation of the T_m).

For radioactively-labeled DNA or RNA probes an example of stringent hybridization conditions is hybridization in a solution containing denatured probe and 5x SSC at 65°C for 8-24 hours followed by washes in 0.1x SSC, 0.1% SDS (sodium dodecyl sulfate) at 50-65°C. In general, the temperature and salt concentration are chosen so that the post hybridization wash occurs at a temperature that is about 5°C below the T_M of the hybrid. Thus for a particular salt concentration the temperature may be selected that is 5°C below the T_M or conversely, for a particular temperature, the salt concentration is chosen to provide a T_M for the hybrid that is 5°C warmer than the wash temperature. Following stringent hybridization and washing, a probe that hybridizes to the tumor antigen associated antibodies , as evidenced by the presence of a signal associated with the appropriate target and the absence of a signal from the non-target nucleic acids, is identified . It is further appreciated that in determining probe specificity and in utilizing the method of this invention to detect tumor antigen specific antibodies, a certain amount of background signal is typical and can easily be distinguished by one of skill from a specific signal. Two fold signal over background is acceptable.

This invention is further illustrated in the Experimental Details section which follows. This section is set forth to aid in an understanding of the invention but is not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

EXPERIMENTAL DETAILS:

EXAMPLE 1: **EARLY DETECTION OF OVARIAN AND BREAST CANCER**

Methods and Materials:

Antibodies extracted from breast cancer tumors, which were bound to tumor cells, have been shown to bind to a Mucin, specifically Muc-1, molecule. Further analysis showed that it the antibody binds to the protein core/backbone

of Mucin-1. No genetic mutations were found in the protein sequence for Mucin-1. The behavior of a normal protein (non mutated) as a foreign antigen was attributed to the deglycosylation of the protein core. Deglycosylation of the protein core may be the reason that Mucin-1 is reconginzed as a foreign antigen. The peptides P1-P3 were designed to cover the immunodominant epitope of the tandem repeat of Mucin-1 which has several configurations and is common to both breast cancer and ovarian cancer. The peptides that have been used were derived from the tandem repeat of the MUC-1.

OC-P1 = SGSGHGVTSAPDTR-NH_2, (SEQ ID NO. 1)
OC-P2 = SGSGAPDTRPAPGSTAP-NH_2,(SEQ ID NO. 2)
OC-P3 = IISAVVGIL-NH_2 (SEQ ID NO. 3)

The first two are soluble in water, the last is very hydrophobic and needs very low pll for disloving it. The first two were bound to Biotin on their Ac side (for technical reasons) the third was not. The level of purity of OC-P1 and OC-P2 is high >95%. OC-P3 is only ~60% pure.

Results:

The cohort from which the preliminary data was collected included 15 women that under went surgery due to suspected OC. Control samples (n=21) were made available from the blood donor services. Antibodies were looked for both in serum (IBTs) and in supernatant fluids (IBTc) following culture of lymphocytes for 6-7 days. Three peptides used were designed based on the published sequences of MUC-1 and HER-2neu.

As can be seen in Table 1, based on histopathological findings, 11/15 were found to be malignant. None of the controls had any reaction in the serum or after in vitro culture to any of the three peptides. IBTs and IBTc showed reactivity against at least one peptide in 10/11 cases. When the assays were combined, 11/11 (100%) were antibody positive. All 4 benign cases were IBTs positive, but only one sample was (weakly) positive using IBTc. Though the number of

individuals tested is too small for large scale statistical analysis and predictions, the data seems to indicated that by using both assays all tumors could be identified via peptide specific antibodies providing a convenient and practical tool for testing in populations at high risk for OC. The fact that only 1/4 benign cases were IBTc positive while 10/11 did so in the malignant cases could indicate that the IBTc might help in the discrimination of benign versus malignant cases.

Table 1. **Diagnostic value of the new IBT by diagnostic grading.**

TEST SYSTEM	CANCER	BENIGN	NORMAL
IBTs (using serum)	10/11	4/4	0/21
IBTc (using culture)	10/11	1/4	0/21
IBTs + IBTc	11/11	4/4	0/21

Blood samples were collected from patients undergoing surgery for suspected OC, and from healthy blood donors. Grading was done on the basis of histopathological findings.

Discussion:

The immune responses test for (antibodies) are antigen specific. The antigens chosen for the screen determine the specificity of the test to cancer or to any specific cancer (breast in this case). It has been repeatedly shown that there is a cross reactivity between ovarian and breast cancer, and that some antigens, such as MUC-1, can be used for detection of both. The cross-reactivity is attributed to the fact that both cancers are hormonal sensitive by the same hormones.

Other examples of cancer antigens include HERneu2 in Breast cancer. Women at high risk for BC (in familial BC for example) are able to show, through longitudinal studies that the appearance (or rise) in antibodies against HERneu2 precedes detection by mammography and/or physical examination by hand.

EXAMPLE 2:

The present invention provides a simple and early initial screening or detection of a tumor in a subject via the immune system. As the tumor grows, its suppresive effect on the immune system becomes stronger, thus active secretion of antibodies might be very low or even barely detectable. The present invention uncovers the potential of the humoral arm of the immune system which enables detection of the tumor specific antibodies even if production of antibodies are suppressed *in vivo*. Therefore, acting as a warning signal.

Methods:

Blood and tissue samples (tumor) were taken with consent from ovarian cancer patients. The patients were all women that underwent surgery. Where applicable cystic fluid was colleted.

Whole blood and separated plasma and PBMC were used for the study. Cells were put into culture for 4-6 days with RPMI complete media and 1:200 pokeweed mitogen (PWM) in a 37 C CO_2 incubator. The samples were later tested for antibody anti the peptide using standard peptide ELISA (1ug/well peptide, blocked by BSA).

Results:

The results are recorded as O.D. readings of the ELISA. Each mark or letter (h=healthy or c=cancer) is one sample tested. The statistical analysis is based on a 95% confidence interval.

Figure 1: P3 - One dimensional analysis

The sample distribution of the O.D. results in two groups c=cancer, h=healthy. As demonstrated there is a trend for higher and more specific sensitivity against the peptide among the cancer patients. However, one dimensional analysis does not appear to be a sufficient as an indicator of the patients having cancer at this size sample.

Figure 2: P3 and P2 - Two dimensional analysis

One run of both peptides. The computer was able to frame (x:.5; y:.52) the normal (=healthy) samples (3-5 known high positive controls at a range of 0.6 - 0.65 O.D. were run together with 3-5 known negative sample controls at a range of 0.25-0.3 O.D., which are not shown in Figure 2) By doing so only very few cancer samples were considered normal and visa versa.

Figure 3A-3B: P2 and P1 and P3 - two dimensional analysis

The samples studied were run at groups of <100 each. Two-dimensional analysis of two different peptide ELISAs run on the same set of samples. Figures 3A of p2 and p3 and Figures 3B of p1 and p3 shows that a clear line devides the h=healthy patients from the c=cancer patients. The patients to the right of the line are those in which the immunologic profile indicates the presence of ovarian or breast cancer. While, patients to the left of the line indicates a patient is healthy. The ratio between the O.D. reading of p1 and p3 or p2 and p3 determines the diagnosis.

Table 2. Results of three dimensional linear classification

Group	Classified into group.					
	Re-substitution			Cross-Validation		
	Normal	Benign	Cancer	Normal	Benign	Cancer
48 Normal	41	7	0	40	8	0
56 Benign	2	32	22	3	25	28
38 Cancer	2	14	32	2	16	20

Table 2 shows the linear classification of the combined 3 peptides. As demonstrated there is a high resolution using both methods of statistical calculation.

EXAMPLE 3 **EARLY DETECTION OF PROSTATE CANCER**

Prostate specific tumor antigen (PSA) rises markedly due to the tumor growth. It is not clear whether there are tumor antigenic / tumor antigenic differences between the PSA in the serum in normal levels and the PSA produced by the tumor or because of it. Based on other examples (such as the MUC-1 in breast cancer) there could be such differences. Then the detection of antibodies against it could serve as a follow-up of PSA from serum of prostate cancer patients will be used as tumor antigen peptides/segments form it, or cells from a cell line from the tumor...).

Sera and supernatant fluid from new method cultures will be tested for PSA specific reactivity. It is expected that there will be higher antibody levels in the serum of some of the patients then in the population of normal controls, but a much higher proportion of them will have anti PSA antibodies in the culture fluid.

Virus encoded tumor antigens from tumor inducing viruses (both DNA and RNA viruses, even though these tumor antigens are more commonly found with relation to RNA viruses as far as expression of the cell membrane.

For example gp70 (which is also found on chemically induced tumors) from the virus envelope glycoprotein. Purified gp70 will be used as tumor antigen (or defined peptides/segments form it, or infected cells expressing it). Sera and supernatant fluid form new method cultures will be tested for gp70 specific reactivity. It is expected that there will be higher antibody levels in the serum of some of the patients than in the population of normal controls, but a much higher proportion of them will have anti gp70 antibodies in the culture fluid.

EXAMPLE 4. **MULTIPLE TUMOR ANTIGEN ASSAY**

An tumor antigen (on some type of a carrier) is inside the tube during the incubation period of the culture (blood culture). The presence of the tumor antigen serves mainly as a "detector" and "binder" (for ligand) for the tumor

antigen specific antibodies that are present (or being produced) in the culture. It could also serve at the same time as a specific stimulator (specific)of the specific segment of the immune process that is taking place in the tube.

The tumor antigen may be in the tube prior to blood drawn in (or cells added) or added at any time-point during the culture. (Thus it could be in contact during the culture:days, hours or minutes). The tumor antigen can be attached to (or bound to): 1) The tube itself either above the level of the culture liquid, at the level of the culture liquid, or below the level of the culture liquid; 2) bound to a nitrocellulose strip; 3)bound to any synthetic carrier (plastic of all sorts); 4) bound to beads, microspheres, etc; 5) be part of a "dry chemistry" (or more exact "dry immunochemistry" system [closed or open]). The surfaces of all the above, (especially beads), can be smooth or grooved or in any other form of shape that will increase its surface area.

The tumor antigen can be bound to all the above carriers directly or via carriers, "arms" and other methods that put a distance between the Ag and the "carrier's" surface. The tumor antigen can be applied to the "carrier" at any shape or size: ring around the tube, a line, a dot, a shape of a + or + or P or any other letter or shape.

The tumor antigen can be attached to the carrier at one given concentration or at several concentrations, (either as discreet "sites" or as a continuous (or gradient). The tumor antigen can be a few tumor antigens - as more than one tumor antigen can be applied to one tube (or carrier or bead or beads, etc.). The different tumor antigens can be applied together (as a mixture) or in groups or individually. When applied to more than one spot, they can be applied using the same shape of mark or using a different one for each tumor antigen.

The detection of the antibodies that bind to the tumor antigen can be done by any of the "developing" and detection systems that are known to date (or that will be found later. The developing reagents can be in the tube initially or added at a

later time. A very simple method will be to finish the development stage towards the end of the culture period or after the end of it all together. The development is either by direct binding of "tagged" antibodies or by competition assays. The "tag" can be an enzyme, a metal, a color colloid, (or fluorescent, luminescent etc.)

EXAMPLE 5:

Alloantibodies:

Even after the most careful matching of blood types for blood transfusions the donor may elicit an immune response against some minor histocompatibility antigens, thus alloantibodies will be formed. In most cases this phenomenon does not have major pathological effects. In some patients the response is strong and can cause hemolysis of the donor's blood putting the recipient at greater danger. These alloantibodies disappear months after the transfusion making the next blood matching for the same individual patient inadequate (the two blood are mixed in vitro to detect adverse reactions but if the antibodies are not there any more the two blood will appear compatible). If the person carries the memory for producing the alloantibodies these will emerge shortly and possibly be lethal shortly after the second transfusion, putting his/her life at risk.

Using the new culture method a sample of the recipient's blood is cultured and the supernatant fluid is tested for its reactivity with the proposed blood units, thus enabling the choice of units that will not lyse or cause any other damage in vivo.

EXAMPLE 6:

To increase the chances of a transplanted tissue or organ to survive and to be accepted by the recipient two parameters are dealt with in parallel: 1. the immune system is suppressed prior to the transplantation and following it. 2. The tissues are matched in the best way possible. Two main methods for matching : 1. Antigenic / structural, i.e. major and minor transplantation antigens are identified and the best match sought after. 2. Biological / immunological i.e. testing in vitro

the reaction of the blood (or its components the leukocytes and the plasma) of the recipients with the cells of the donor.

Since much of the antibody repertoire of the immune system is not active and secreted in a given time much of the potential humoral response to the transplant cannot be seen by the existing methods. The new culture method exposes these antibodies and testing the culture-supernatant or cells in vitro with the donor's cells can expose dangers prior to the final choice of donor and rejection in vivo that could be stronger the original anticipated and prepared against.

EXAMPLE 7: **DETECTION OF ANTIBODIES TO H. PYLORI**

Correct interpretation of antibody patterns in seropositive individuals is important in understanding the immunodominant epitope mapping. Individuals exposed to an antigen usually mount a broad humoral immune response to many antigens. Early detection of exposure to antigens or the confirmation of the exposure is possible based on this assay.

Strains of Helicobacter pylori have been associated with type B chronic gastritis and may play an etiologic role in peptic ulcer disease. Moreover, recent evidence suggests a role for H. pylori in the development of gastric carcinoma. Methods for detecting infection by H. pylori include the use of a urea breath test and detection of anti-H.pylori antibodies in patients' sera by techniques such as immunofluorescence, latex agglutination, and complement fixation assays. However, the most commonly used systems are those based on the enzyme-linked immunosorbent assay (ELISA). Most ELISA systems use antigens prepared from whole-cell protein extracts (acid-glycine or whole-cell sonicates).

This bacteria has many antigenic / immunogenic epitopes or structures. The whole bacteria, or some of its proteins, carbohydrates, mucins, or peptides can serve as antigen for the antibody detection. H. pylori strains originally isolated from U.S. patients are disrupted by sonication. After centrifugation, the supernatants are pooled and plated in 0.05 M carbonate buffer (pH 9.6) onto flat-

bottomed microtiter wells (Immulon-2, Dynatech Laboratories, Alexandria, Va.) as test antigen.

Antibodies to H.pylori can be measured separately for IgG, IgA, , IgE, IgD, and IgM by an enzyme immunoassay method. The absorbance readings can be converted to reciprocals of the end-point titers. The high- molecular-weight, cell-associated antigen preparation contains at least two proteins in the molecular-weight range of 400,000-700,000. Thus looking for an antibody (or antibodies) against Helicobacter pylori means using the whole immunogen or segments (peptides) of it as the antigen for the detection systems and tests.

The presents of antibodies against H. pylori is an indication of a peptic ulcer background that which would lead to proper treatment of H. pylori infection thus enabling the healing of the mucosal lining of the digestive system.

EXAMPLE 8: **EARLY DETECTION OF ANTIBODIES OF HTLV**

Method and Materials:

Heparinized blood samples from blood bank donors in were used in this experiment. One ml. Of blood was mixed with 2 ml. Of complete media and the mitogens. Tubes were cultured in 37°c humidified CO_2 incubator. The culture fluid was tested for the presence of virus-1 specific antibodies using commercial ELISA. The results are O.D. readings. The control readings were Neg:0.006, pos;0.370.

Results:

Table 1: Serology versus the new test for HTLV-1 antibodies:

	Serology	New-test
1.	<0.030	0.230*
2.	<0.030	0.070
3.	<0.030	0.160

4.	0.054	0.300*
5.	<0.030	0.102
6.	<0.030	0.043
7.	<0.030	0.052
8.	<0.030	0.090
9.	0.048	0.311
10.	<0.030	0.170*
11.	<0.030	0.023
12.	<0.030	0.099

Sample numbers 1, 3, 4, 9, and 10 were seronegative to HTLV-I yet antibodies against the virus were detectable after culture. Samples marked by an asterisk (*) were further confirmed (the cutoff for that run was 0.100 O.D.)

EXAMPLE 9: **EARLY DETECTION OF SPECIFIC ANTIBODIES OF HCV EXPOSURE**

Correct interpretation of antibody patterns in seropositive individuals understanding the immunodominant epitope mapping. There are numerous immunologic epitopes encoded by the HCV genome, both in structural and nonstructural proteins. Infected individuals usually mount a broad humoral immune response to many antigens; however, no specific antibody pattern has been identified that can differentiate recovery from persistent HCV infection. In most prospectively followed patients with acute infection, antibodies to core (c22-3) and NS3 (c33-c) antigens develop before and at higher titers than antibodies to other antigens, although in some cases antibodies to NS4 (c-100-3) or to NS5 antigens appear first. No seroconversion pattern has been shown to predict outcome of infection. Immunodominant epitopes have been found within the N-terminus of the core antigen (aa 20 to 34) and within the NS4 region (aa 1712 to 1731), the latter corresponding recombinant antigens (C-22-3 and C-100-3, respectively). Most, if not all, chronically infected patients have antibodies against the hypervariable 5' end of the E2/NS1 gene product (gp70).

Furthermore, biopsy proven chronic hepatitis has been documented both in patients with seemingly acute self-limited hepatitis C and in anti-HCV positive blood donors with persistently normal ALT levels. The documented occurrence of transfusion-associated hepatitis (TAH) C virus in recipients of anti-HCV negative blood, the frequent loss of HCV-RNA from the serum of patients under interferon treatment with subsequent reappearance of viremia after interferon withdrawl, and the finding of HCV-RNA in the liver of patients with seronegative chronic hepatitis C suggest that neither normalization of ALT, disappearance of anti-HCV antibodies nor disappearance of serum HCV-RNA necessarily imply complete recovery. Therefore, it seems likely that persistent infection (whether active or quiescent) occurs in the majority of HCV-infected individuals.

Although the mechanism(s) underlying HCV persistence are unknown, some observations suggest potential strategies by which HCV might regulate its lytic potential and avoid detection and elimination by the host's immune system. Since HCV does not replicate through a DNA intermediate, its persistence cannot be explained in terms of latency through integration of viral genome into the host genome.

The currently available diagnostic armamentarium of HCV infection includes serologic assays based on recombinant protein and/or synthetic peptide-based antibody capture assays, gene amplification techniques for detection of HCV-RNA sequences in serum or liver, and immunohistochemical and "in situ" hybridization techniques for detection of HCV-antigens or HCV-RNA sequences in tissue. Tissue based techniques are in the early stages of development and available in only a limited number of research laboratories. The relationship of HCV detection assays to the clinical course of hepatitis C is shown in Fig. 2-2.

First generation anti-HCV assays detected antibodies to a single recombinant antigen (C-100-3), which corresponds to a small portion of the C'-terminus of

NS3 and nearly all of the NS4 gene product expressed in yeast as a fusion protein.

The most extensively evaluated second generation tests (EIA-2) detect antibodies to the core recombinant protein C-22-3 and to a recombinant protein, C-200, representing a composite of recombinant proteins including C-33$_c$ (NS3) and C-100-3 (NS4). EIA-2 increases seroconversion rates in acute, transfusion-associated, or sporadic NANB hepatitis by 10-20% and shortens the window period between disease onset and seroconversion by a mean of 8 weeks; in 70-80% of cases, EIA-2 can detect anti-HCV within 4 weeks of disease onset.

The most extensively evaluated "confirmatory assay" is a second generation recombinant immunoblot assay (RIBA-2, Chiron Corporation, Emeryville, CA.). This assay consists of a nitrocellulose strip tp which recombinant HCV proteins 5.1.1(NS4), C-100-3(NS4), C-33-c(NS3) and C-22-3(core), have been blotted as discrete bands, along two levels of human IgG and superoxide dismutase (SOD) control.

The most consistent IgM anti-HCV response is directed against the nucleocapsid (core) antigen, and in some instances is the first marker of active anti-HCV seroconversion. Although IgM reactivity is short-lived compared to IgG and hence IgM antibody can be utilized as an acute phase marker of HCV infection.

Sequence diversity in both structural and non-structural coding regions of different HCV isolates can result in false-negative PCR reactivity. Primers specific for the highly conserved 5'UTR should be used to avoid missing viremia due to sequence heterogeneity. The use of nested PCR (double OCR with nested primers), frequently used to increase sensitivity and to avoid the hybridization step, greatly increases the inherent risk of contamination and may yield amplificator products of the expected size that are nonspecific.

Method and Materials:

Heparinized blood samples from individuals that are folowed due to a high risk of HCV infection were used in this experiment. One ml. of blood was mixed with 2 ml. of complete media, and the mitogen(PWM) was added. Tubes were cultured in 37°c humidified CO_2 incubator. The culture fluid was tested for the presence of HCV specific antibodies using ELISA kit (Minoliga). The results are O.D. readings. The cut-off for this run was 0.392. The study center considers positive those that are at lease 1.2 of that cut-off (-0.470). The confirmation test was for structural and non structural antibodies (in serum only).

Results:

All samples in Table 2. are subjects from high risk groups for Hepatitis C virus. All antibodies were Hepatitis C virus specific.

Table 2.

MADA#	Serology	New-test	Confirmation
5312	0.155	0.262	-
5313	0.449	0.500	-
5314	0.410	0.557	+
5315	0.159	0.165	un
5316	0.342	0.553	-
5317	0.502	1.015	-
5318	0.516	0.761	+/-
5319	0.246	0.498	+/-
5320	0.430	0.366	-

un = undetermined

Sample 5319 is a clear example of better detection via culture than via serology. Since the cutoff was close to 0.4 O.D. Sample 5314 is also a clear positive (also confirmed) only after culture. Further studies in population of individuals with an initial positive or boarder line ELISA in one test.

Results:

Table 3.

Donor No.	O.D.ratio in serum	O.D.ratio in supernatant	Confirmation
5328	3.12	3.29	-
5329	1.93	1.88	+
5330	1.11	0.92	-
5331	5.51	2.14	+
5332	5.56	5.98	+
5321	1.70	1.99	-
5322	1.08	2.35	-
5323	0.58	0.30	un
5324	0.61	1.32	-
5325	1.10	2.01	-
5326	1.31	2.05	+
5327	3.62	5.90	+

un = undetermined

Confirmation was only done using the structural and non structural protein antibody-test in the serum. Sample 5322, 5325, 5326 are examples of a positive diagnosis only after culture.

EXAMPLE 10: **ELISPOT DETECTION**

Method and Materials:

This invention contemplates an antibody-detection system such as ELISPOT in which cells which actively secrete the specific antibody are detected and may be counted.

Method and Materials:

Inactivated whole SIV was applied onto a nitrocellulose membranes in a 96 well plate. SIV was washed off and the membranes blocked with 0.2% non fat

powdered milk. At limiting dilutions the cells were added to the wells and incubated for 6 hours. The plates were rinsed 5X with PBS-Tween and then developed using antibody α Human IgG (which also binds Monkey IgG) and a non soluble product of a color reagant to visualize the dots where SIV pecific antibodies (secreted by the cells) bound to the SIV in the well. The purple-blue spots were counted under x4 (or x10) magnification. The frequency and number of SIV specific cells was calculated by standard methods of limiting dilution.

Herparinized blood samples from Sooty Mangabey monkeys that have already been identified as silent carriers of SIV, and from some positive and negative controls were used in this experiment. One ml. Of blood was mixed with 2 ml. Of complete media. Each of the mitogens was tested in a separate tube. Tubes were cultured in 37°c humidified CO_2. The culture fluid was tested for the presence of SIV specific antibodies using an in-house SIV ELISA kit with disrupted and inactivated whole SIV particles were used as antigen. The results are O.D. readings.

Results:

Table 5.

Frequency of anti SIV Ig secreting B cells

Monkey Anti SIV Serology	Anti SIV Ig B Cells/ Total B Cells	Anti SIV Ig B Cells 10^8 PBL
Sooty mangabey +	1/300	3,333
Sooty mangabey +	1/218	4,587
Rhesus macaque +	1/600	1,666
Rhesus macaque +	1/400	2,500
Rhesus macaque -	0	0
Sooty mangabey -	0	0

SIV specific B cells which actively secrete antibodies can be detected only in seropositive monkeys

Table 6.

Comparison of ELISPOT with ELISA titers from Cultures with PWM from Seronegative Mangabeys Monkeys

Monkey	ELISA O.D.	ELISPOT Frequency	No.of cells/10^B
1	0.003 ± 0.006	<0/10^6	0/10^6
2	0.342 ± 0.037	1/2168	461/10^6
3	0.105 ± 0.042	1/9775	102/10^6
4	0.409 ± 0.063	1/5866	171/10^6
5	0.045 ± 0.021	<1/20,000	<50/10^B

Monkey # 1 was a true negative for SIV. (Cells were taken from a seronegative rhesus macaque.) No SIV specific cells were detected.

While no SIV antibody producing cells are detectable in seronegative monkeys even if they are (silent) carriers the SIV primed B cells can be detected by ELISPOT after culturing. They also have detectable antibodies in the culture. The frequency and number of the SIV specific cells in a "silent carrier" is much lower than in a seropositive SIV carrier.

EXAMPLE 11: **WESTERN BLOT DETECTION**

The new method was also used with Western blot as the detection method. From this work the following was shown for SIV and HIV: 1. All positive serum samples were positive post culture too; 2. No bands were lost due to the culture phase; 3.In some seropositive cases the culture stage led to additional positive bands; 4.In seronegative high risk individuals (and monkeys) that had positive ELISA tests clear bands of all the major HIV (and SIV) proteins showed reactivity. Some samples had only part of the bands, usually env and core.

EXAMPLE 12. **EARLY DETECTION OF HEPATITIS B (HBV)**

There is window period of at least several weeks between infection and antibody formation in vivo up to detectable levels in current diagnostic tests (usually ELISA). Screening a population for the presence of HBV specific antibodies (using ELISA) both in scrum/Plasma and in the supernatant fluid of the culture using the new method and kit will show that: All seropositives will be positive by the new method too (100% relative specificity) While some of the seronegative samples will be positive using the new method and kit. At a later time point these new-method-positives will seroconvert thus clearly showing the true specificity of the assay(not false positive results in the discordant samples) and its superior sensitivity. Other ways of proving the presence of the infection can be used such as PCR.

Antibody to Hepatitis B core antigen (anti-HBc) - All blood transfused in the U.S. is screened for anti-HBc. Although initially introduced as a surrogate test for non-A-non-B hepatitis, it is now used as a "lifestyle" indicator to detect protential carriers of other viruses (such as HIV) and as a test to detect the rare Hepatitis B carrier who is antigen negative but still infectious (anti-HBc alone [without anti Hbs] has been shown to have the potential to transmit Hepatitis B). The current test for anti-HBc is an ELISA.

EXAMPLE 13. **EARLY DETECTION OF HPV**

Women at very early stages of the HPV infection might not be detected using a cervical smear test. The value of antibody testing is not very clear but even if antibodies are not produced in easily detectable levels the new method might offer an opportunity to try screening and very early detection using the new method and kit.

EXAMPLE 14. **EARLY DETECTION OF CYTOMEGALOVIRUS (CMV)**

The prevalence on CMV exposure in the adult population is quite high, and not all blood units are screened for CMV. Yet, new born babies, and

immunosuppressed individuals (such as cancer patients under radiation or chemotherapy, or pstients after a tissue transplant) could get a deadly post transfusion infection if given CMV infected blood. Thus some 40% of the blood is screened for CMV as only CMV negative blood can be given to these patients. Some of these CMV-negative units do transmit CMV leading to grave situations.

CMV- Although most Cytomegalovirus (CMV) infections are subclinical, this virus is associated with three major clinical states: 1., congenital defects after intranterine infection; 2., neonatal infection and death; 3., CMV pneumonia in immunosuppressed hosts, especially those undergoing bone marrow transplantation. About half the U.S. population has been infected, and a significant proportion of these will still harbor the virus (latent) even though they are well and have antibodies. The virus can be activated in the host under a variety of conditions. CMV is also transmitted by blood transfusion when given to CMV negative recipients. Because of its clinical significance, blood transfusions given to susceptible CMV negative patients are often screened for antibody to CMV, and if positive, such units are withheld from transfusion. Perhaps 1/3 of units transfused in the U.S. are screened for CMV antibody so that adequate stock of CMV-negative blood could be available if needed.

EXAMPLE 15:

As to the "predictive value" of the culture assay . In over 60 other "silent carrier" monkeys that did not seroconvert during the same length of follow-up, all the O.D. readings post culture were positive but low (~ X2 of the negative control of the assay). Figures 1A-1F.

Materials and Methods:

Test for serum and post-culture antibodies at given intervals (on the first 2-4 months after the initial positive culture-Antibody test once a week, if no seroconversion occurred till then one can be considered a "silent carrier" and be followed once a month or so). An increase in O.D. reading (or ratio) of more

than 50% in two successive measures (or 100% increase in one) is an indication of a seroconversion in the near future.

Discussion:

Sequential O.D. reading (y axis) for SIV specific antibodies of cultures blood samples taken at different time points (x axis). Figures 1B-1F each represents 1 monkey. In addition to these 5 examples of seroconversion more than 60 other monkeys (Mangabeys) were followed for 2 years. Their level of *in vitro* SIV antibody production remained always low positive. None of those 60 seroconverted during the follow up. There were 3 additional monkeys whose *in vitro* level of SIV antibodies rose only at the seroconversion sample. SIV antibody level rose markedly and steadily prior to seroconversion. Thus predicting the seroconversion months ahead of time.

The importance of this prediction stems from several factors: a) The shift from Th1 to Th2 response against the virus (HIV for example) leads not only to seroconversion but also to a pathological sequela at a future date; b) the seroconversion process is usually preceded by a surge in viral replication. Therefore, anti viral treatment, which would not be recommended at the latent preseroconversion state would be very efficient at that stage (it might even be able to revert the process and prevent the seroconversion; c) there could be a recommendation to use at the point of pre-seroconversion some treatment that will suppress the humoral arm or have other beneficiary effects on the immune balance so as to enter the seropositive state at an immune set-up that will offer a longer asymptomatic period, better coping with the surging infection. Figures 1A-1F shows that the level of O.D. reading on ELISA , post culture, can differentiate between true negative, sero positive, and silent infection/ exposure.

EXAMPLE 16. PREDICTIVE VALUE OF ASSAY IN A HUMAN

Table 7

Anti HIV Ab ELISA O.D

Time post exposure	Negative control c/o	Serology	IVAP	PCR and ISH
1 week	0.061	0.020	0.015	nd
2 weeks	0.054	0.038	0.148	-
4 weeks	0.034	0.008	0.209	±
2 months	0.088	0.022	0.428	+
3 months	0.065	0.021	0.490	+
5 months	0.057	0.032	0.212	

Follow-up of a needle stick injury

HIV specific antibodies from a subject in the in vitro antibody production (IVAP) test were detected using GENELAVIA and further confirmed on ELAVIA (both by Diagnostic Pasteur). PCR was run in two different laboratories and ISH (In Situ Hybridization) in a third one. A positive result means positive in all three.

As shown in Table 6, a rise in O.D. (=antibody level) of culture cells preceeds a clear positive PCR test, i.e. preceeds a rise in infectious load. Detection of HIV antibodies is at two weeks.

What is claimed is:

1. An *in vitro* method for the detection of tumor antigen associated antibodies in a sample obtained from a subject, comprising the following steps:

a) obtaining a whole blood sample from the subject;

b) incubating the whole blood sample in a culture in the presence of a mitogen with or without antigen containing media, so as to induce polyclonal and specific activation of lymphocytic cells leading to tumor antigen associated antibody production and the expression of tumor antigen specific antibodies;

c) exposing the resultant culture of step b) to an tumor antigen, thereby allowing an antigen-antibody immune complex to form; and

d) detecting the antigen-antibody immune complex of step c); wherein the presence of antigen specific antibodies is indicative of the subject being with a tumor.

2. The method of claim 1, wherein the culture of step b) results in a supernatant, and the supernatant is exposed to an tumor antigen, thereby allowing an antigen-antibody immune complex to form.

3. The method of claim 1, wherein the culture of step b) results in cellular fraction which is exposed to an antigen, thereby allowing an antigen-antibody immune complex to form.

4. The method of claim 1, wherein the mitogen is an activator of lymphocytic cells.

5. The method of claim 4, wherein the mitogen is pokeweed mitogen, a lectin, a bacterial endotoxin, a tumor antigen, lipid A, or a lymphokine.

6. The method of claim 5, wherein the mitogen is pokeweed mitogen.

7. A method for screening a subject having ovarian or breast cancer, comprising the following steps:

a) obtaining a whole blood sample from the subject;

b) incubating the whole blood sample in a culture in the presence of a mitogen with or without antigen containing media, so as to induce polyclonal and specific activation of lymphocytic cells leading to tumor antigen associated antibody production and the expression of tumor antigen specific antibodies;

c) exposing the resultant culture of step b) to an tumor antigen, thereby allowing an antigen-antibody immune complex to form; and

d) detecting the antigen-antibody immune complex of step c); wherein the presence of tumor antigen specific antibodies is indicative of the having ovarian or breast cancer.

8. The method of claim 7, wherein the culture of step b) results in a supernatant, and the supernatant is exposed to an tumor antigen, thereby allowing an antigen-antibody immune complex to form.

9. The method of claim 7, wherein the mitogen is an activator of lymphocytic cells.

10. The method of claim 9, wherein the mitogen is pokeweed mitogen, a lectin, a bacterial endotoxin, a tumor antigen, lipid A, cytokine, or a lymphokine.

11. The method of claim 9, wherein the mitogen is pokeweed mitogen.

12. A method of detecting a subject with a tumor , comprising the following steps:

a) obtaining a whole blood sample from the subject;

b) incubating the whole blood sample in a culture in the presence of a media containing a mitogen with or without antigen, so as to induce polyclonal and specific activation of lymphocytic cells leading to tumor antigen associated antibody production;

c) exposing the cells so as to separately recover nucleic acid sequences;

d) contacting the resulting nucleic acid sequences with single-stranded labeled oligonucleotide primers, the primers being capable of specifically hybridizing with nucleic acid sequence of a tumor antigen associated antibody , under hybridizing conditions;

e) amplifying any nucleic acid sequences to which a pair of primers hybridizes so as to obtain a double-stranded amplification product; and

i) detecting the presence of the amplification product, the presence thereof being indicative of the presence of the tumor antigen .

13. The method of claim 12, wherein the nucleic acid sequence is DNA, RNA or cDNA.

14. The method of claim 12, wherein the single-stranded oligonucleotide primers are labeled with biotin.

15. The method of claim 12, wherein the single-stranded oligonucleotide probes are labeled with fluorescein.

16. The method of claim 12, wherein the marker is alkaline-phosphatase.

17. The method of claim 12, wherein the mitogen is an activator of lymphocytic cells.

18. The method of claim 12, wherein the mitogen is pokeweed mitogen, a lectin, a bacterial endotoxin, a virus, lipid A, or a lymphokine.

19. A kit for the detection of specific tumor antigen associated antibody from a subject, comprising: a container for collecting whole blood samples, wherein the container contains a media containing mitogen with or without antigen, effective to induce polyclonal and specific activation of lymphocytic cells leading to antibody production and an assay for the detection of the specific tumor antigen associated antibody.

20. The kit of claim 19, wherein the assay is an enzyme linked immunosorbent assay, or an immunofluorescence assay.

21. The kit of claim 19, wherein the container is made of a plastic, glass, or metal material.

22. The kit of claim 21, wherein the container is a test tube or a flask.

23. The kit of claim 22, wherein the container is vacuum sealed.

24. The kit of claim 19, wherein an tumor antigen is bound to the container.

25. The kit of claim 24, wherein multiple tumor antigens are bound to the container.

26. A method of detecting a subject exposed to an antigen , comprising the following steps:

a) obtaining a whole blood sample from the subject;

b) incubating the whole blood sample in a culture in the presence of a media containing a mitogen with or without antigen, so as to induce polyclonal and specific activation of lymphocytic cells leading to antibody production;

c) exposing the cells so as to separately recover nucleic acid sequences;

d) contacting the resulting nucleic acid sequences with single-stranded labeled oligonucleotide primers, the primers being capable of specifically hybridizing with nucleic acid sequence of a antigen specific antibody , under hybridizing conditions;

e) amplifying any nucleic acid sequences to which a pair of primers hybridizes so as to obtain a double-stranded amplification product; and

i) detecting the presence of the amplification product, the presence thereof being indicative of the presence of the antigen .

27. The method of claim 26, wherein the nucleic acid sequence is DNA, RNA or cDNA.

28. The method of claim 27, wherein the single-stranded oligonucleotide primers are labeled with biotin.

29. The method of claim 27, wherein the single-stranded oligonucleotide probes are labeled with fluorescein.

30. The method of claim 27, wherein the marker is alkaline-phosphatase.

31. The method of claim 27, wherein the mitogen is an activator of lymphocytic cells.

32. The method of claim 31, wherein the mitogen is pokeweed mitogen, a lectin, a bacterial endotoxin, a virus, lipid A, or a lymphokine.

33. The method of claim 32, wherein the mitogen is pokeweed mitogen.

34. A kit for the detection of specific antibodies resulting from exposure to an antigen from a subject, comprising: a container for collecting whole blood samples, wherein the container contains a media containing mitogen with or without antigen, effective to induce polyclonal and specific activation of lymphocytic cells leading to antibody production and an assay for the detection of the specific antigen antibody.

35. A method of detecting a subject infected with a virus , comprising the following steps:

a) obtaining a whole blood sample from the subject;

b) incubating the whole blood sample in a culture in the presence of a media containing a mitogen with or without antigen, so as to induce polyclonal and specific activation of lymphocytic cells leading to antibody production;

c) exposing the cells so as to separately recover nucleic acid sequences;

d) contacting the resulting nucleic acid sequences with single-stranded labeled oligonucleotide primers, the primers being capable of specifically hybridizing with nucleic acid sequence of a virus specific antibody, under hybridizing conditions;

e) amplifying any nucleic acid sequences to which a pair of primers hybridizes so as to obtain a double-stranded amplification product; and

i) detecting the presence of the amplification product, the presence thereof being indicative of the presence of the virus

FIG. 1


Frequency of P3
Symbol Category Symbol Category
Ovarian Cancer
C
Healthy
H
FREQUENCY
No. Patients
50
40
30
20
10
0.1
0.3
0.5
0.7
0.9
1.1
1.3
P3 Midpoint
O.D. Reading on Peptide ELISA

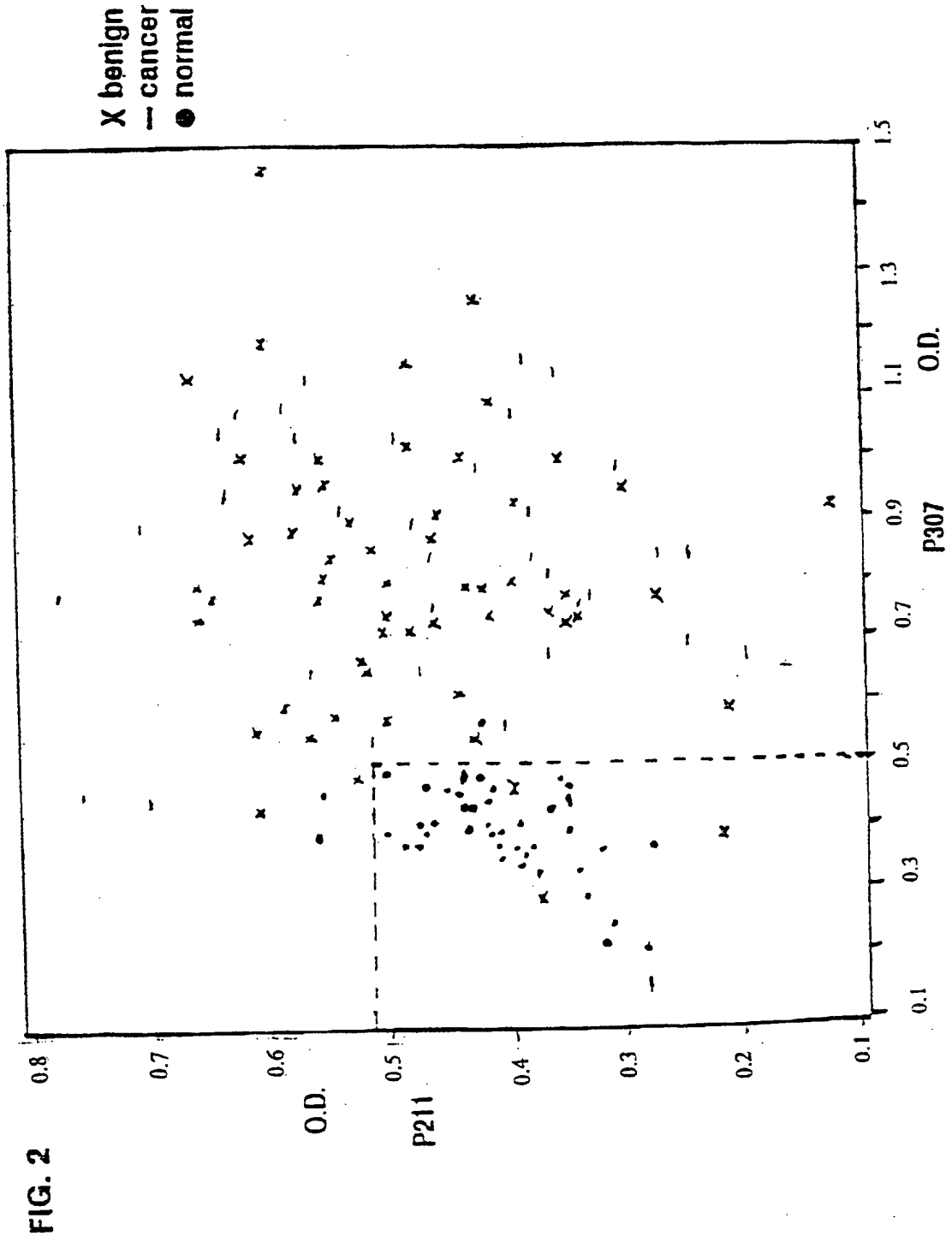
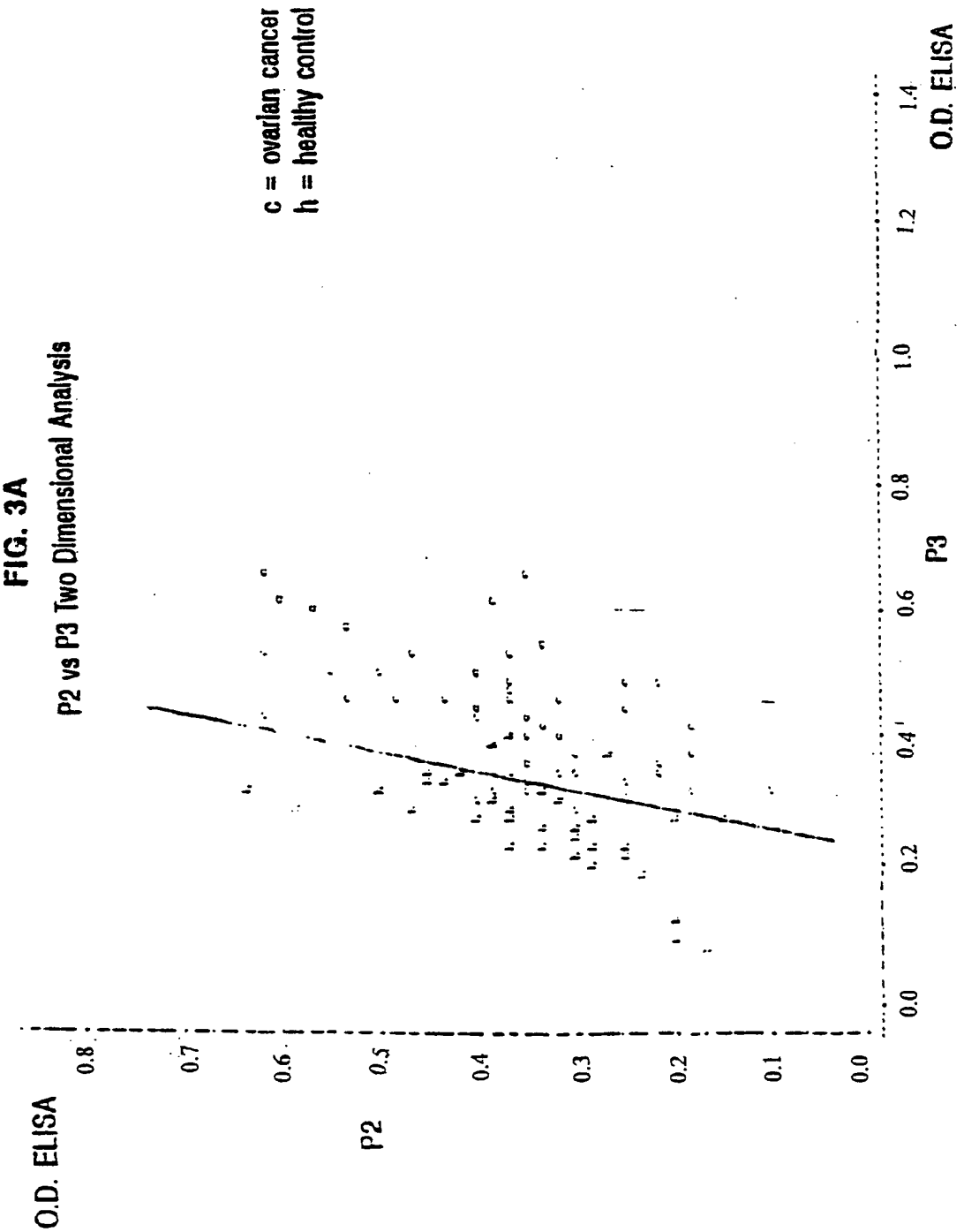

X benign
— cancer
● normal
O.D.
P211
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
O.D.
P307
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1

FIG. 2

FIG. 3A

P2 vs P3 Two Dimensional Analysis



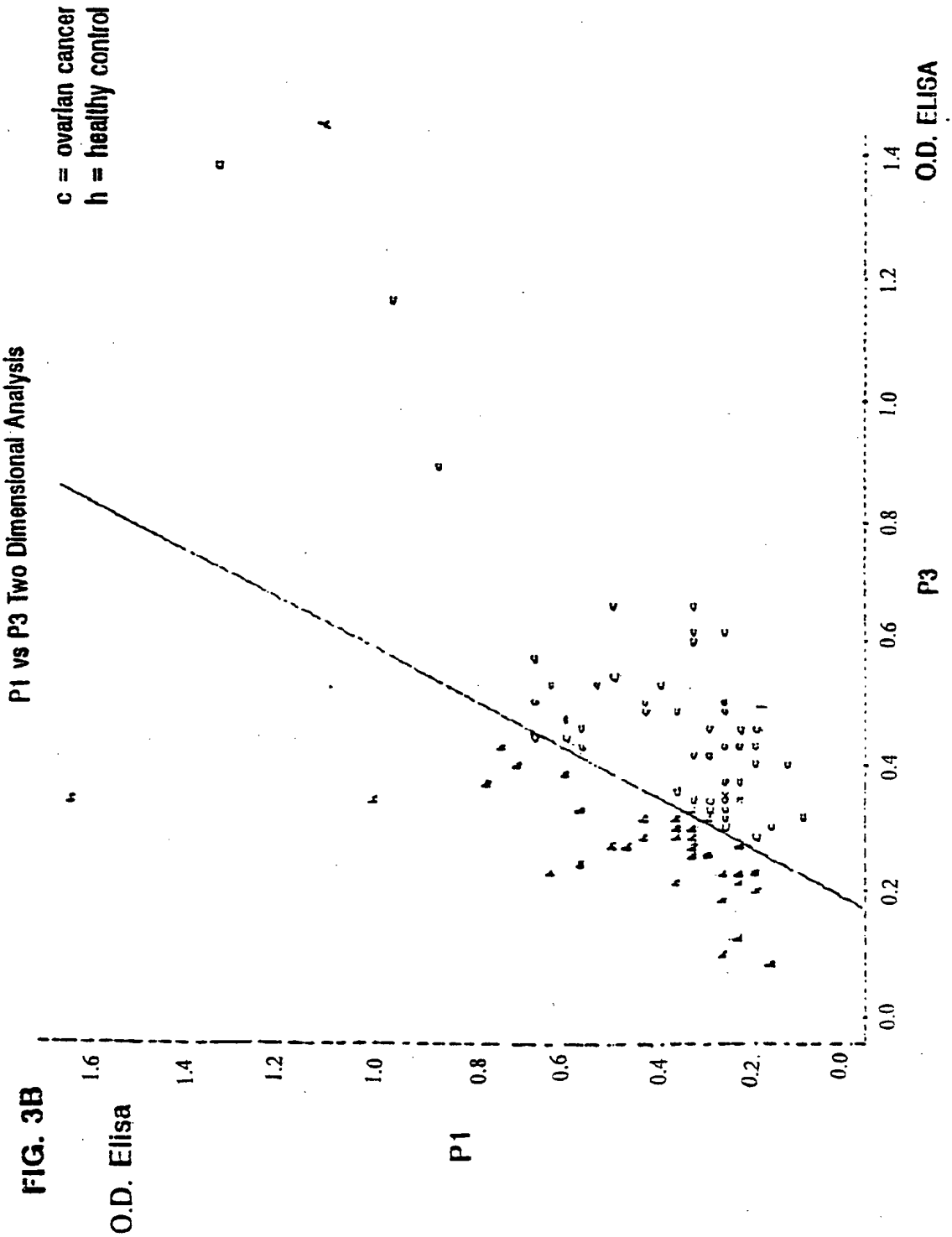

P1 vs P3 Two Dimensional Analysis
c = ovarian cancer
h = healthy control
O.D. Elisa
P1
P3
O.D. ELISA
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6

FIG. 3B

FIG. 4A

The New/Culture Assay Detects SIV Specific Antibodies In Seronegative Mangabeys

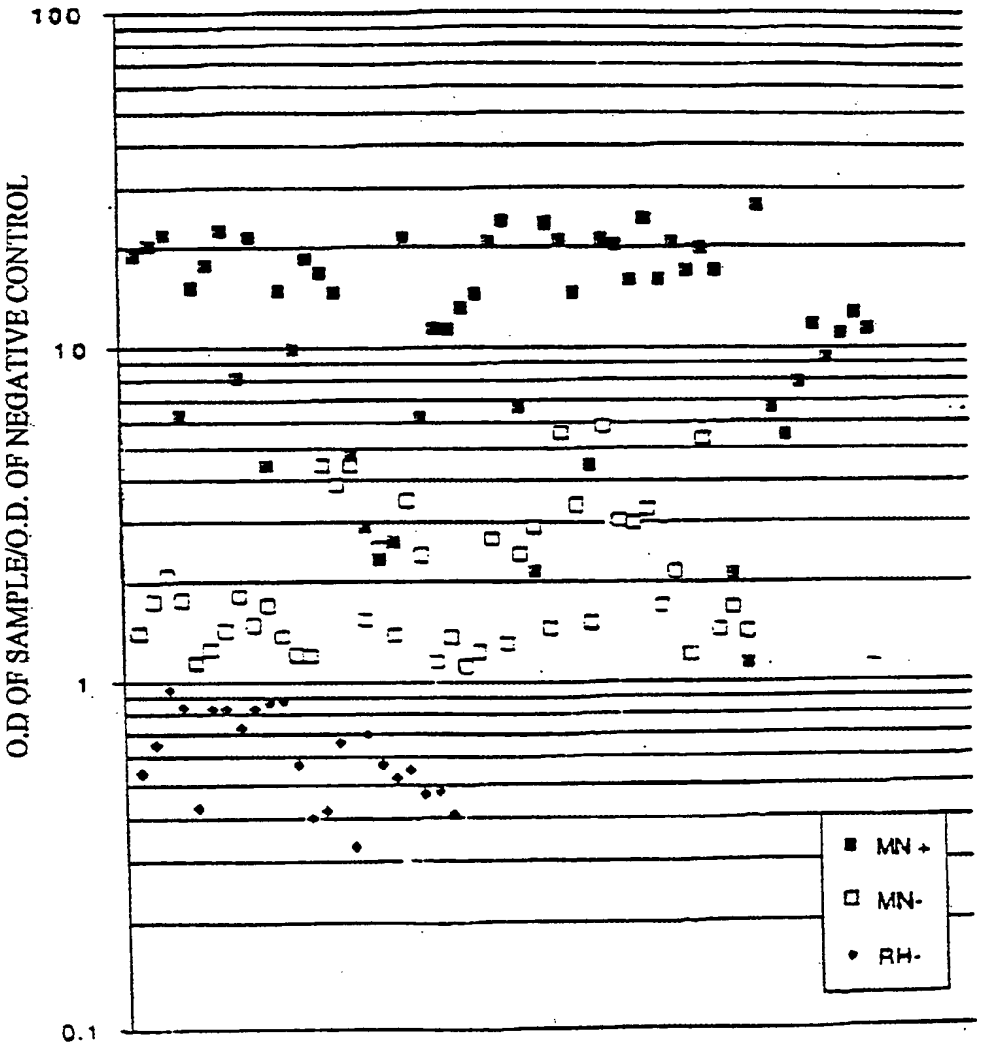


FIG. 4B

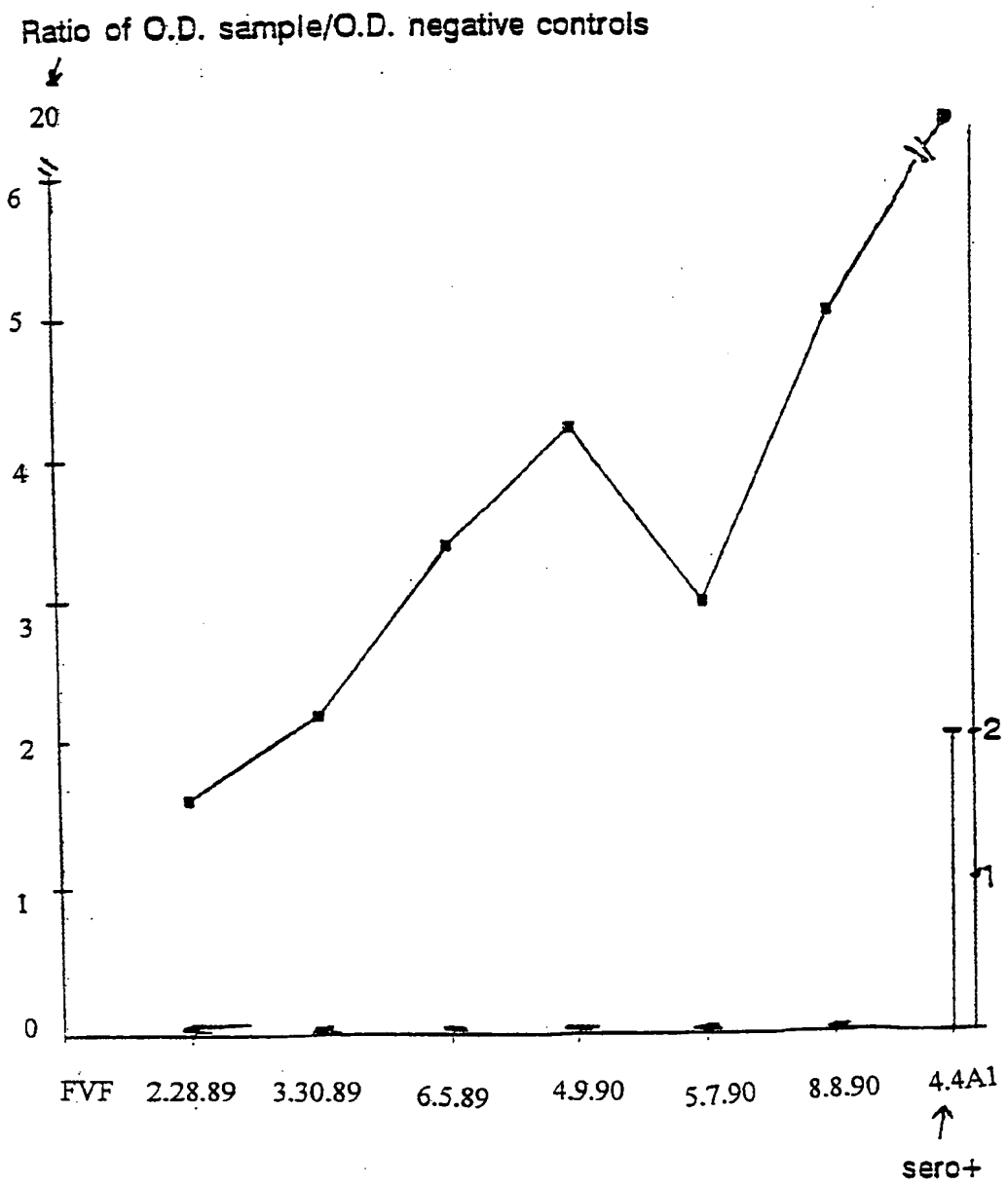

Ratio of O.D. sample/O.D. negative controls
20
6
5
4
3
2
1
0
2
1
FVF
2.28.89
3.30.89
6.5.89
4.9.90
5.7.90
8.8.90
4.4A1
sero+
T = serum Ab
= culture Ab

FIG. 4C

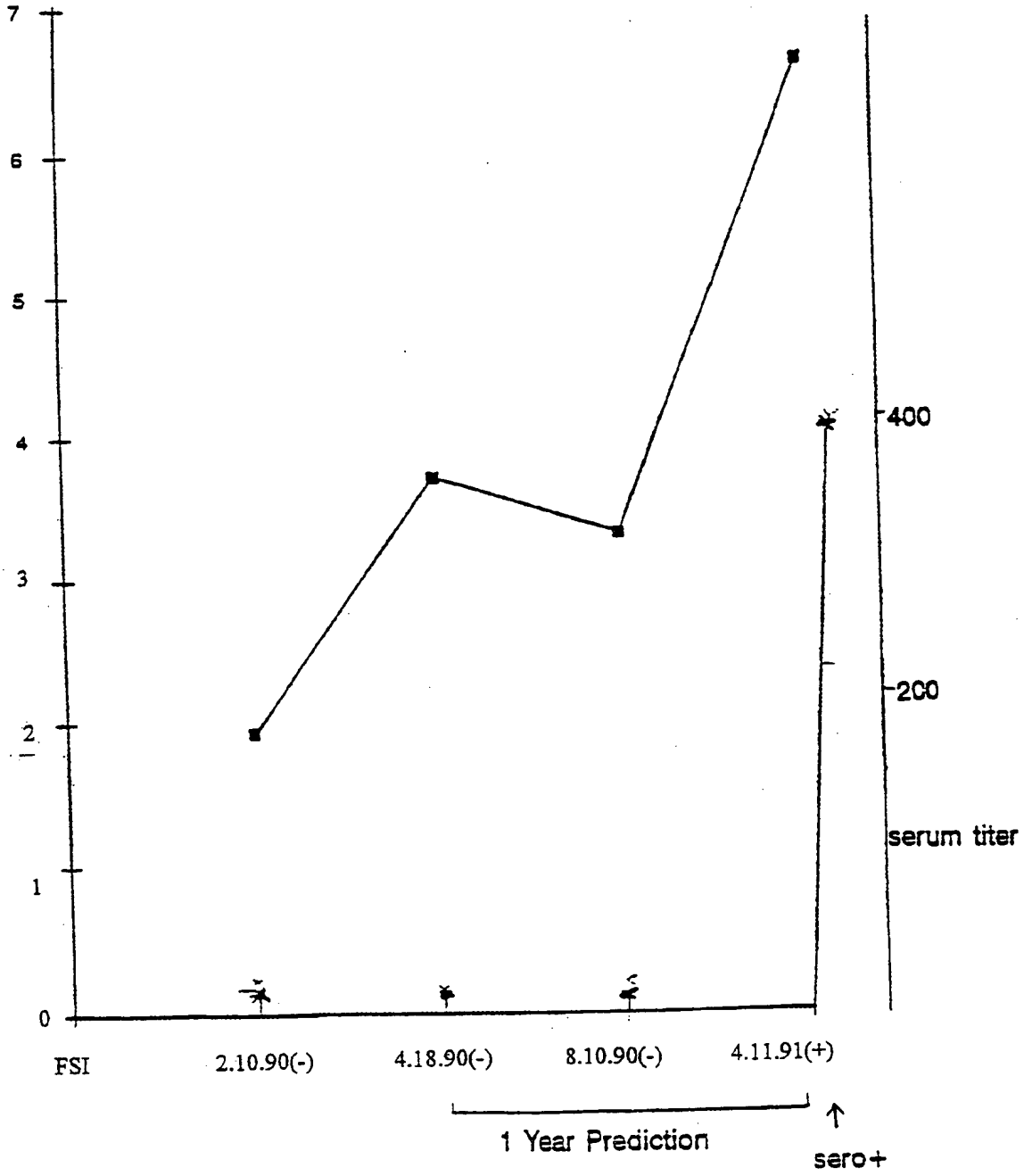
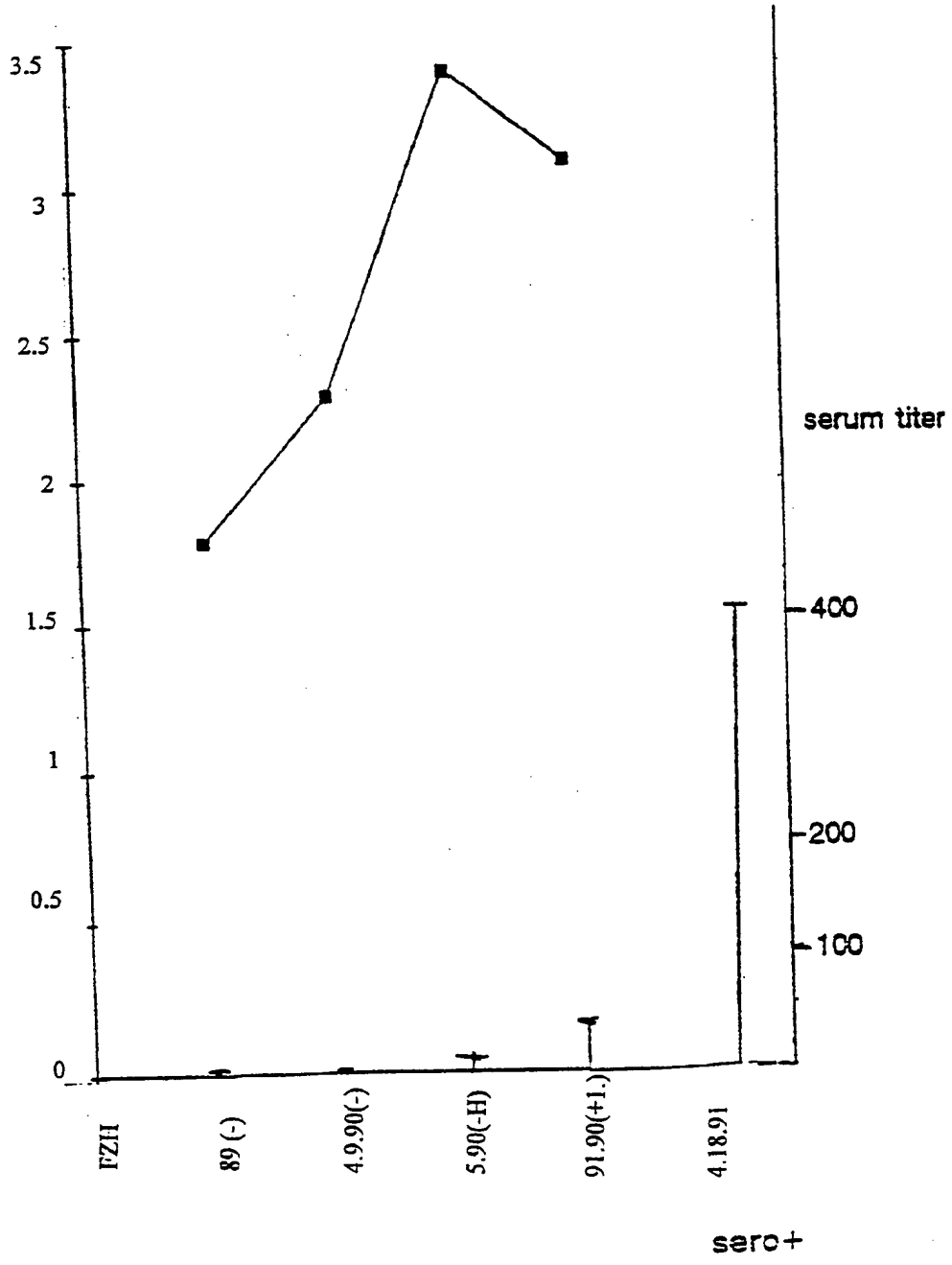

7
6
5
4
3
2
1
0
400
200
serum titer
FSI
2.10.90(-)
4.18.90(-)
8.10.90(-)
4.11.91(+)
1 Year Prediction
sero+

FIG. 4D


3.5
3
2.5
2
1.5
1
0.5
0
serum titer
400
200
100
FZH
89 (-)
4.9.90(-)
5.90(-H)
91.90(+1.)
4.18.91
sero+

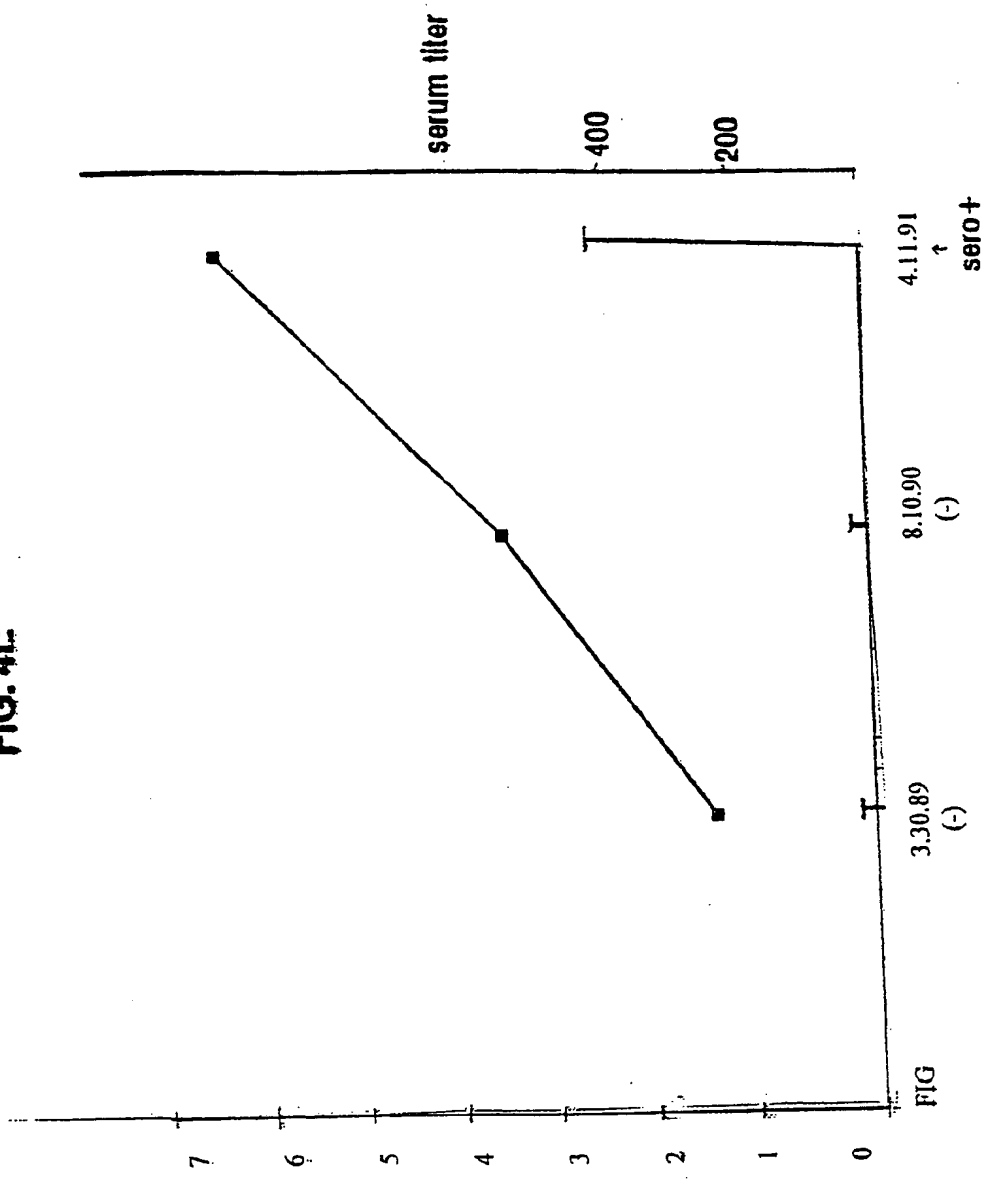
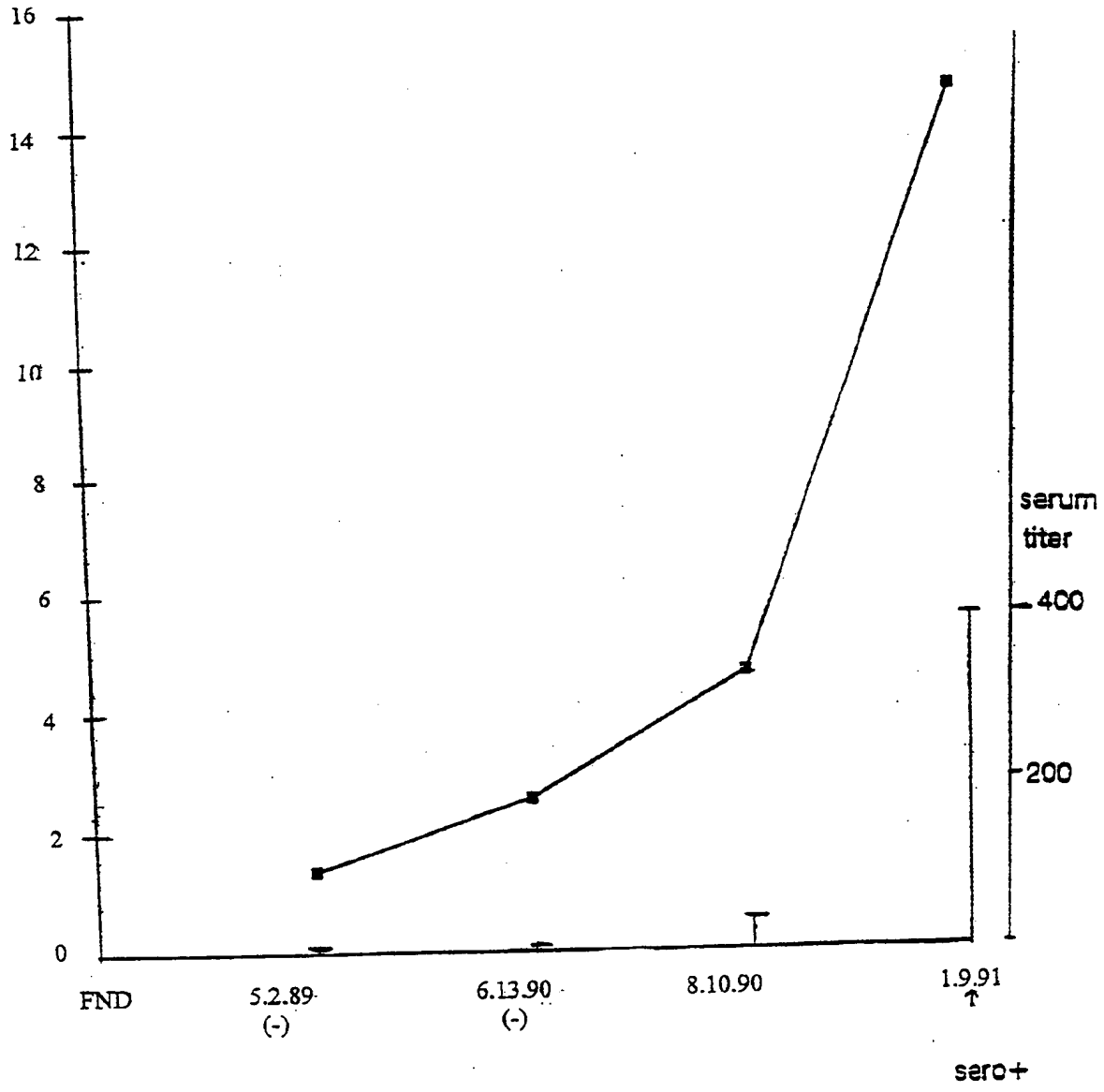


FIG. 4E

FIG. 4F


16
14
12
10
8
6
4
2
0
serum titer
400
200
FND
5.2.89
(-)
6.13.90
(-)
8.10.90
1.9.91
sero+

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International Bureau

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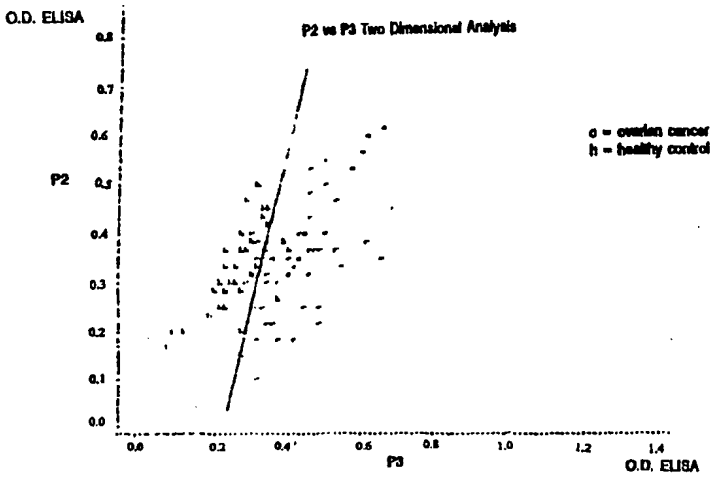

(57) Abstract

This invention provides a method of detecting a subject having ovarian or breast cancer, comprising the following steps: a) obtaining a whole blood sample from the subject; b) incubating the whole blood sample in a culture in the presence of a media containing a mitogen with or without antigen, so as to induce polyclonal and specific activation of lymphocytic cells leading to antibody production; c) exposing the resultant culture of step b) to a specific tumor antigen, thereby allowing an antigen–antibody immune complex to form; d) detecting the antigen–antibody immune complex of step c); wherein the presence of tumor antigen associated antibody is indicative of the subject having the cancer.

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FI	Finland
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GA	Gabon
GB	United Kingdom
GE	Georgia
GH	Ghana
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IL	Israel
IS	Iceland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgyzstan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakstan
LC	Saint Lucia
LI	Liechtenstein
LK	Sri Lanka
LR	Liberia
LS	Lesotho
LT	Lithuania
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
MK	The former Yugoslav Republic of Macedonia
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TM	Turkmenistan
TR	Turkey
TT	Trinidad and Tobago
UA	Ukraine
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US	United States of America
UZ	Uzbekistan
VN	Viet Nam
YU	Yugoslavia
ZW	Zimbabwe

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Interr nal Application No
PCT/IL 97/00381

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IPC 6 G01N33/569 G01N33/574 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 93 11435 A (COHEN STEVEN J) 10 June 1993 see claim 1	1-11, 19-25,34
Y	EP 0 203 403 A (ASAHI CHEMICAL IND) 3 December 1986 see claim 7	1-11, 19-25,34
A	WO 93 14189 A (LUDWIG INST CANCER RES) 22 July 1993 see the whole document	1-11, 19-25,34
A	DE 195 15 219 A (PECHER GABRIELE DR) 31 October 1996 see figure 1	1-11, 19-25,34

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 June 1998	12. 06. 98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hoekstra, S

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intern ıal Application No
PCT/IL 97/00381

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 88 10314 A (UNIV VERMONT) 29 December 1988 see the whole document ---	12-19, 21-33
A	EP 0 653 493 A (TAISHO PHARMA CO LTD ;LTT INST CO LTD (JP)) 17 May 1995 see the whole document see example 4 -----	12-19, 21-33

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int. ational application No.
PCT/IL 97/00381

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/IL 97/00381

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-11, 20 AND 34 ENTIRELY; 19 AND 21-25 PARTIALLY

   Method for detecting antibodies to tumor antigens or tumor associated antigens in whole blood samples from a subject comprising incubating the whole blood, (culturing the blood cells) with a mitogen, exposing the result to the antigen and detecting antibody-antigen complex formation.

2. Claims: 12-18,
   26-33 AND 35 ENTIRELY; 19 AND 21-25 PARTIALLY

   Method for detecting antibodies to antigens in whole blood samples from a subject comprising incubating the whole blood sample with a mitogen (culturing the blood cells), recovering nucleic acids from the cells and exposing the nucleic acid to labeled oligonucleotide primers encoding part of the gene encoding the antigen specific antibody, amplifying any nucleic acids to which a pair of primers hybridize and detecting the amplification product.

INTERNATIONAL SEARCH REPORT

...formation on patent family members

Inter nal Application No
PCT/IL 97/00381

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